AN INVESTIGATION OF WNT FUNCTION IN RADIAL GLIAL NEURAL PROGENITOR CELLS IN THE ZEBRAFISH HYPOTHALAMUS

by

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A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Neuroscience

Department of Neurobiology and Anatomy

The University of Utah

August 2015

The University of Utah Graduate School

STATEMENT OF DISSERTATION APPROVAL

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ABSTRACT

The mammalian hypothalamus has recently been shown to support the continual generation of new neurons, even in adults. The zebrafish also generates neurons in the adult. I have undertaken a multifaceted investigation into the regulation of the cells that support this homeostatic addition of neurons to the hypothalamus, radial glial cells, by the Wnt/β-Catenin signaling pathway. In Chapter 1, I describe the nature of neural stem cell populations and their development. I go on to describe Wnt signaling pathways and their potential regulation of neurogenesis through mitogenic affects on stem cell populations and promotion of differentiation.

In Chapter 2, I present the first comprehensive analysis of the expression of *wnt* genes in the vertebrate central nervous system. Using three developmental time points, we have discovered that *wnt* genes are redundantly expressed throughout development. This analysis will shed light on potential drivers of Wnt signaling pathways that could regulate stem cell function, differentiation of neurons or glia, axonal path-finding, as well as tissue morphogenesis during central nervous system development. Although this investigation was carried out in the zebrafish central nervous system in particular, these data could offer insight into drivers of Wnt signaling pathways in the mammalian central nervous system as well.

In Chapter 3, I present a large body of work investigating the nature of radial glial progenitor cells during postembryonic homeostasis in the zebrafish hypothalamus. I

demonstrate that these neural progenitors are multipotent and support the regeneration of themselves as well as dopaminergic neurons. I go on to determine that Wnt/β-Catenin signaling is not necessary for these phenomena. Interestingly, we have also revealed that in fact, Wnt β-Catenin signaling needs to be kept low in radial glial cells to maintain them as multipotent neural progenitors.

In Chapter 4, I discuss the roles for Wnt/β-Catenin signaling in the regulation of differentiation and proliferation of this neural progenitor population. I present several avenues for future investigation that would further shed light on the nature of radial glial cell proliferation in the postembryonic hypothalamus, including candidate signaling pathways that are likely to regulate RG activation. I go on to discuss Wnt/β-Catenin signaling inhibition in RGs as a necessity for their maintenance. Finally, I discuss the mechanisms by which Wnt/β-Catenin signaling may be inhibited specifically within the RG population in the hypothalamus.

To my parents Betty Lou Duncan and Jon Duncan, and to everyone I have shared a rope with.

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ACKNOWLEDGEMENTS

I have grown incredibly as a scientist, alpinist, dirtbag, and human during my time here in Utah. I am indebted to Rich Dorsky and Tatjana Piotrowski for patiently mentoring me while I was both a young and impatient graduate student, and currently as an old and impatient graduate student. I am overjoyed to have had so many adventures, with incredible friends, in the mountains and at the crags in Utah, Mexico, California, Nevada, Canada, Cuba, Oregon, Wyoming, China, and Argentina. I tip the hat to my trusty rig, Roxanne, who almost always starts when I turn the key. Foremost I am incredibly thankful for the infinite love and support of my incredible parents.

CHAPTER 1

INTRODUCTION

1.1. Overview

Stem cell and progenitor populations are often thought to be stimulated to divide, or 'activated' to leave a quiescent state and proliferate, by Wnt/β-Catenin signaling. However, there are few studies that have investigated the dependence of radial glial neural progenitors in-vivo on Wnt/β-Catenin signaling. This thesis has elucidated the nature of Wnt/β-Catenin signaling's regulation of hypothalamic radial glial cells

- by identifying the *wnt* genes that are expressed in the hypothalamus through development,
- by determining that radial glial cells are multipotent neural progenitors that support regeneration,
- by determining that Wnt/β-Catenin signaling is not necessary for radial glial function during neurogenesis, self-renewal, maintenance, or regeneration
- by determining that Wnt/β-Catenin signaling needs to be kept low in radial glial cells to maintain them.

In addition to a comprehensive analysis of *wnt* gene expression patterns in the zebrafish CNS, presented in Chapter 2, we have endeavored to label radial glia in the zebrafish hypothalamus genetically to study their potential in normal homeostasis in Chapter 3. In Chapter 3, we go on to combine the postembryonic labeling of this neural progenitor population with Wnt/β-Catenin signaling perturbations to determine if in fact Wnt/β-Catenin signaling is necessary for stem cell 'activation' during normal homeostasis and during regeneration. Surprisingly, we have found that radial glial cells do not depend on Wnt/β-Catenin signaling for any type of division or maintenance, but

instead require that Wnt signaling remain inhibited to maintain this multipotent self-renewing population of neural progenitors. We have found that Wnt/β-Catenin signaling does not have a generalizable role in stem cell populations, but in fact, Wnt/β-Catenin signaling's most conserved function is to promote differentiation.

1.2. Homeostatic neurogenesis is widespread in postembryonic animals

The existence of functional neural stem cells in numerous brain regions in adult mice and humans has been appreciated for about two decades (Gould, 2007). These neural stem cells generate newborn neurons that integrate into the existing circuitry of adult animals, most notably the hippocampus and olfactory epithelia, and help animals to generate new spatial memories and distinguish between new odors, respectively (Imayoshi et al., 2008; Lazarini and Lledo, 2011). More recently it has become clear that neurogenesis is supported in the mammalian hypothalamus (Cheng, 2013; Kokoeva et al., 2007; Maggi et al., 2014) as well as other brain regions (Chapouton et al., 2007). In the teleost zebrafish, there are many brains regions that support neurogenesis and neural stem cells are also capable of proliferating and repairing injury in the central nervous system (CNS) of the zebrafish (Baumgart et al., 2012; Kroehne et al., 2011), in contrast to the relatively nonregenerative mammalian CNS (Kyritsis et al., 2014). We have used the posterior recess of the hypothalamus of the zebrafish as a model to study the regulation of neural progenitor cells in-vivo during homeostatic neurogenesis and during regeneration. The biological function of homeostatic neurogenesis in the zebrafish hypothalamus is currently unclear.

1.3. Development of neural stem cells from neuroepithelial cells

Neuroepithelial cells generate neurons during CNS development in mammals and zebrafish and are transformed into radial glial (RG) cells, which continue to generate neurons, as development proceeds (Fig. 1.1.) (Barry et al., 2014). RG cells are a heterogeneous group of cells. Classically defined by their polarized morphology and glial nature, these criteria are still valid (Barry et al., 2014; Gotz and Barde, 2005). Traditionally RG cells were though to be a transient cell type that is responsible for considerable CNS neurogenesis and patterning (Barry et al., 2014). RGs divide to give rise to neural progenitors that in turn migrate radially along their basal processes to take up residence away from the ventricular zone during neurogenesis. However, these cells are in fact retained in many ventricular zones of the CNS in vertebrates, most notably in the dentate gyrus of the hippocampus (Malatesta and Gotz, 2013), but including Bergmann glia in the cerebellum, Mueller glia in the retina, tanycytes in the hypothalamus, and RG in the ventricular zone of the spinal cord and cerebral cortex (Barry et al., 2014), after development has ended. Notably tanycytes, Mueller glial cells, and the RGs in the hippocampus also maintain their proliferative nature during post-embryonic life (Kokoeva et al., 2007; Ming and Song, 2011; Wang et al., 2012).

RG cells are often labeled with s100β, glutamine synthetase (GS), brain lipid binding protein (BLBP), members of the *Hes/Her* family of Notch signaling transcriptional affecters including *her4.3*, glial fibrillary acid protein (GFAP), and *nestin* (Chapouton et al., 2011; Marz et al., 2010; Yamaguchi et al., 2000). RG cells do not always express all of these markers, the first indication of the heterogeneous nature of this cell type (Pinto and Gotz, 2007). Finally, in the mammalian sub-ventricular zone

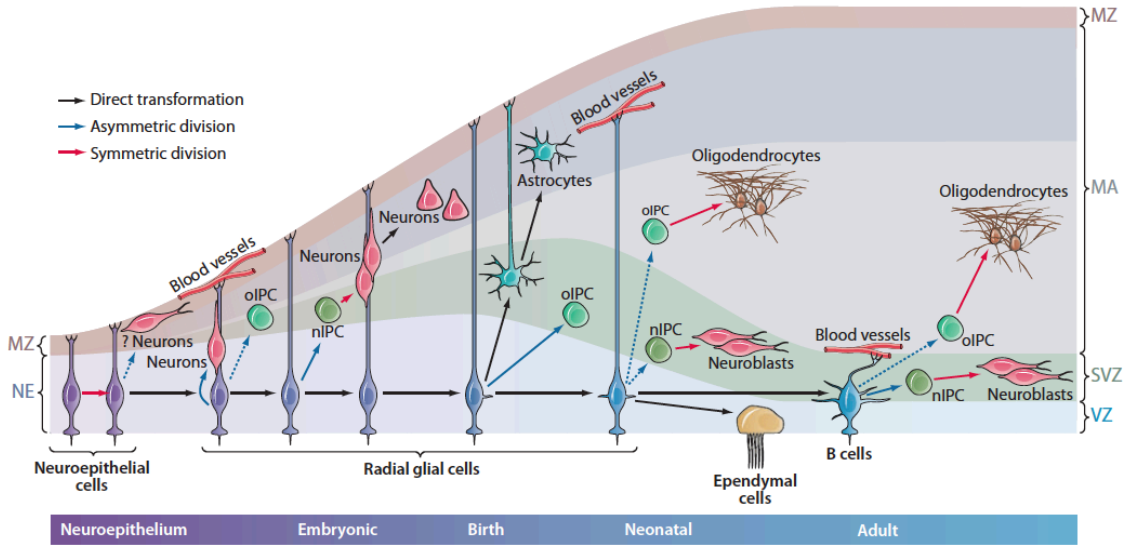

Figure 1.1. Radial glial cells are generated from neuroepithelial cells. Early in development, neuroepithelial cells generate neurons. Neuroepithelial cells are transformed into RG cells as development proceeds. In the SVZ, these RG cells further transform into astrocytic B-cells, the resident stem cell population in the lateral ventricles. In the hippocampus and other CNS proliferative zones, RG cells are maintained and act as stem cells. These RG cells are present in the hypothalamus where they are also called tanycytes. (Kreigstein and Alvarez-Bullya, 2009, used with permission)

(SVZ) of the lateral ventricles, neuroepithelial cells are ultimately transformed into astrocytes (Kriegstein and Alvarez-Buylla, 2009) called B-cells which function as the stem cells driving olfactory neurogenesis, which only further documents the complexity of homeostatic neurogenesis in the vertebrate brain.

1.4. The hypothalamus

1.4.1. Hypothalamic function

The hypothalamus is the most ventral diencephalic structure and is incredibly conserved through evolution (Bedont et al., 2015; Tessmar-Raible et al., 2007). The

hypothalamus is the functional link between the CNS and the endocrine system regulating heart rate and blood pressure, body temperature, fluid and electrolyte balance, appetite and body composition, sleep cycles, as well as the glandular secretions of the stomach and intestine, sexual maturity, and aggression. The hypothalamus is able to exert these effects on the whole body by signaling to the pituitary, which then releases effector hormones into the blood stream. The hypothalamus secretes neurohormones that start and stop the secretion of pituitary hormones. The neurohormones the hypothalamus is able to release include, anti-diuretic hormone, corticotropin-releasing hormone, gonadotropin-releasing hormone, growth hormone-releasing hormone and growth hormone-inhibiting hormone, oxytocin, prolactin-releasing hormone or prolactin-inhibiting hormone, thyrotropin releasing hormone. As such, the hypothalamus' function is crucial for proper biological control of the animal (Kandel and Jessel).

In addition to efferent control of physiological processes, the hypothalamus receives feedback from the body and functions as feedback regulator of many physiological processes. In particular, the hypothalamus is able to respond to the adipose-derived hormone leptin, via the leptin receptor that is expressed in the arcuate nucleus. The activation of the leptin receptor in the arcuate nucleus allows the hypothalamus to signal through AGRP/POMC (Varela and Horvath, 2012) neurons to regulate metabolism of sugars and additionally signals the neuroendocrine axes that control growth as a permissive factor (Allison and Myers, 2014), ultimately ensuring that there is not excessive conversion of glucose into adipose tissue that leads to obesity.

1.4.2. Zebrafish hypothalamus

The zebrafish hypothalamus has completed morphogenesis by 5 days post fertilization but does continue to grow throughout the lifetime of the animal. The hypothalamus is the dorsal most diencephalic brain region. The zebrafish hypothalamus contains an elaboration of the 3rd Ventricle, the Posterior Recess (PR). This bilaterally symmetrical tissue contains Radial Glial cells and multiple types of neurons, including serotonergic, dopaminergic, as well as HuC/D+ neurons that reside along the ventricle and that are nonventricularly localized (Fig. 1.2). We have used the zebrafish PR of the hypothalamus in this thesis.

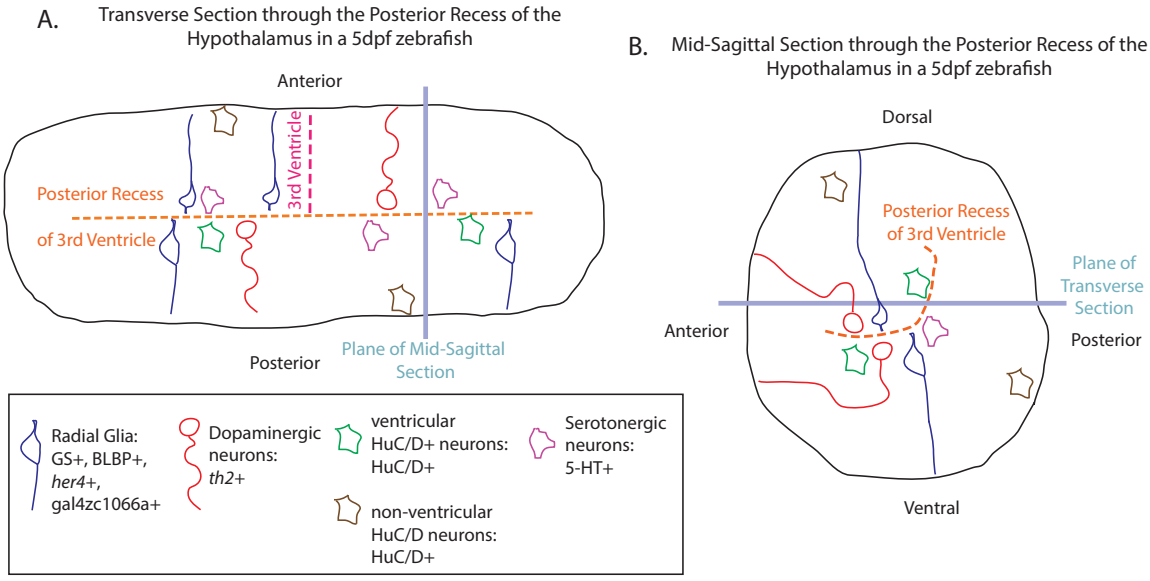

Figure 1.2. The anatomy of the zebrafish Posterior Recess of the hypothalamus at 5dpf. The PR contains Radial Glial cells, as well as multiple classes of neurons. Transverse section of the PR at 5dpf in A, with the 3rd ventricle documented by a pink dotted line and the PR of the 3rd ventricle documented by an orange dotted line, A. Mid-Sagittal section of the PR at 5dpf, in B, with the PR of the 3rd ventricle documented by an orange dotted line. The relative position of each section is delineated by a blue line.

1.4.3. Function of ongoing hypothalamic neurogenesis

Although all behaviors governed by the hypothalamus could be regulated by chronic hypothalamic neurogenesis, numerous studies have demonstrated a specific affect on the regulation of energy homeostasis when hypothalamic neurogenesis is perturbed. It was observed that hypothalamic RG cells, called tanycytes, are proliferative and generate leptin-responsive neurons (Kokoeva et al., 2005). Subsequent studies have shown that postembryonically born neurons in the mouse were generated from radial glial cells within the median eminence of the hypothalamus, and blocking their generation led to deficits in energy homeostasis and feeding behavior (Lee et al., 2012). Taken together, the relevant data suggest that the homeostatic addition of tanycyte-derived neurons to feeding centers in the hypothalamus is critical for the proper regulation of energy balance (Bolborea and Dale, 2013).

1.5. Wnt signaling

1.5.1. Wnt/β-Catenin signaling

The Wnt/β-Catenin signaling pathway, sometimes referred to as the canonical Wnt signaling pathway as it was the first Wnt signaling pathway that was well characterized (McMahon and Moon, 1989; Rijsewijk et al., 1987; Sharma and Chopra, 1976), involves secreted Wnt ligand binding to Frizzled and Low-density lipoprotein Receptor-related Protein 5 or 6 heterodimers (LRP5/6), leading to inhibition of the β-Catenin destruction complex (Angers and Moon, 2009) of which Glycogen Synthase Kinase 3-β (GSK3β) is a critical component. β-Catenin then accumulates cytoplasmically and is transported into the nucleus of the Wnt receiving cell, where β-Catenin complexes

with members of the Lymphoid Enhancer Factor 1 (LEF1)/T Cell Factor (TCF) family of transcription factors. β-Catenin/TCF complexes then bind to TCF/LEF consensus sequences where they activate transcription of Wnt target genes (Fig. 1.3.) (Rao and Kuhl, 2010). Alternatively, in some tissues, Wnt/β-Catenin signaling de-represses Wnt target genes by displacing repressive TCF3 proteins from TCF/LEF sites and replacing TCF3/Groucho complexes that recruit histone deacetylases with transcriptional activator TCF isoforms, including TCF7 and LEF1. In either case the Wnt receiving cell ultimately activates Wnt-responsive genes. It is important to appreciate that many of the proteins in the β-Catenin destruction complex have biological functions beyond simply degrading β-Catenin. In particular, GSK3-β degrades many proteins in a Wnt responsive manner, including cell cycle effectors (Li et al., 2012; Niehrs and Acebron, 2012) and

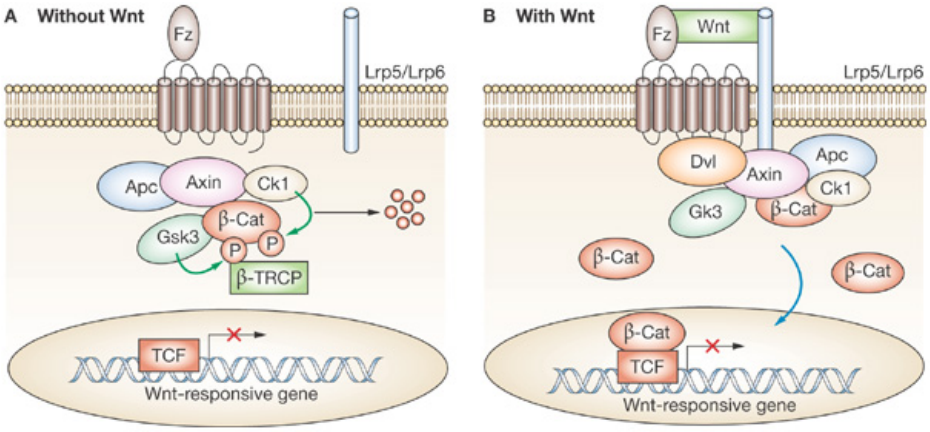

Figure 1.3. Wnt/β-Catenin signaling leads to gene expression changes.
A) In the absence of Wnt presence, the β-Catenin destruction complex functions to phosphorylate and degrade β-Catenin. B) After Wnt ligand binding to Lrp/Fz heterodimers, the β-Catenin destruction complex is perturbed through the recruitment of Disheveled (Dvl). Without its chronic degradation, β-Catenin accumulates intracellularly and eventually is transported to the nucleus where it complexes with transcriptional activator TCF/LEF factors to promote expression of Wnt responsive genes. (Deal, Chad 2009 Nat Clin Pract Rheumatol, used with permission)

Neurogenin. Thus the Wnt/β-Catenin signaling pathway functions to modulate the expression of genes in cells receiving Wnt signals (MacDonald et al., 2009).

1.5.2. β-Catenin-independent pathways

In addition to the Wnt/β-Catenin signaling pathway there are multiple β-Catenin-independent (also called noncanonical) Wnt signaling pathways, so-called because they utilize components of Wnt/β-Catenin signaling without ultimately leading to β-Catenin/TCF-factor-dependent changes in gene expression. There are many different non-canonical Wnt signaling pathways, and it is notable that these pathways can be activated simultaneously to and act independently of Wnt/β-Catenin signaling and additionally that they can attenuate Wnt/β-Catenin signaling in some cases (Bisson et al., 2015). These pathways include: Wnt planer cell polarity pathway and the Wnt-calcium pathway (De, 2011; Veeman et al., 2003) in addition to other less characterized pathways. These pathways most often utilize Disheveled and Frizzled proteins but Wnt ligand binding to Frizzled receptors is not an obligatory feature of β-Catenin-independent Wnt signaling. Downstream of Disheveled activation, these pathways diverge. When Wnt planar cell polarity pathways are activated Frizzled and Disheveled work to polarize the cell by distributing proteins, including Strabismus, Flamingo and Prickle, asymmetrically in the cell, eventually affecting cell migration or convergent-extension tissue morphogenesis. Wnt/Calcium pathways ultimately lead to activation of CamKII and PKC, and potentially to gene expression via NFκB, CREB, or NFAT transcription factors (De, 2011).

1.5.3. Wnt ligands

Classically, Wnt ligands have been thought to act strictly through either β-Catenin-independent or Wnt/β-Catenin signaling pathways; however, it has recently been demonstrated that Wnt5a can activate different Wnt signaling pathways in a context-dependent manner (Li et al., 2009; Mikels and Nusse, 2006), demonstrating the inherently context-dependent nature of Wnt signaling outcomes. Although the *wnt5* and *wnt11* gene families are thought to activate β-Catenin-independent pathways while the *wnt1*, *wnt3,* and *wnt8* gene families of ligands are thought to activate Wnt/β-Catenin signaling the clear classification of these ligands is only possible if you are able to perform true loss of function studies and closely observe the resulting phenotypes (Rao and Kuhl, 2010). Complicating matters further, these genes are often expressed redundantly, as is the case of the *wnt7* gene family of ligands that are co-expressed in the zebrafish nervous system (Beretta et al., 2011). Due to the functional redundancy and co-expression of these ligands, pursuing a loss of function strategy by targeting individual genes is likely too laborious to be a fruitful avenue of investigation unless two or fewer genes are expressed in your area of interest. We have determined that in the zebrafish CNS there is massive redundancy of *wnt* expression in the CNS during development making *wnt* gene loss of function strategies difficult.

1.6. Wnt/β-Catenin regulation of stem/progenitor populations

1.6.1. Embryonic stem cells are regulated by Wnt/β-Catenin signaling

Wnt/β-Catenin signaling regulates ES cells (Sokol, 2011) before it is used to pattern the embryonic body axis along the anterior/posterior axis (Hikasa and Sokol,

2013). Although many studies have generated conclusions that Wnt/β-Catenin signaling stimulates ES cell self renewal, most data suggest that Wnt/β-Catenin signaling promotes differentiation of ES cells. While one group found that inhibiting Wnt/β-Catenin signaling did not affect ES cell renewal or maintenance yet activation resulted in induction of mesoderm lineage genes (Davidson et al., 2012), another group found that Wnt/β-Catenin signaling stimulated differentiating divisions of ES cells; however, the daughter cell that received the highest level of Wnt/β-Catenin signaling maintained pluripotency while the daughter cell with lower levels of Wnt/β-Catenin signaling displayed hallmarks of differentiation (Habib et al., 2013). Further studies have shown that inhibiting Wnt/β-Catenin signaling in murine epiblast stem cells reprograms them to a chimera-competent pregastrula state, and Wnt/β-Catenin inhibition prevents spontaneous differentiation of in human ESCs and murine epiblast stem cells (Kurek et al., 2015), supporting the general model that Wnt/β-Catenin acts on stem populations to promote differentiation. However, other groups have shown that Wnt/β-Catenin signaling inhibits neural differentiation of ES cells and is necessary for the maintenance of pluripotency (Sato et al., 2004).

Wnt/β-Catenin signaling is therefore implicated in both renewal and differentiation of ES cells, differences that might be attributed to dosage of Wnt/β-Catenin signaling between experimental paradigms. It is possible that the absolute levels of Wnt/β-Catenin signaling between experiments are not identical; varying levels of Wnt/β-Catenin signaling have been hypothesized to drive different biological outcomes with lower levels promoting stem cell maintenance and higher levels promoting differentiation or even tumorigenesis (Blanpain and Fuchs, 2009). Additionally

differences in BMP signaling between ES cell populations could account for the different phenotypes- BMP signaling attenuates Wnt/β-Catenin signaling and affects neuroepithelial cell maintenance and proliferation in the dorsal neural tube (Ille et al., 2007). It is very likely that Wnt/β-Catenin signaling is a highly context-dependent regulator of proliferation and differentiation (van Amerongen and Nusse, 2009).

1.6.2. Wnt/β-Catenin signaling in adult epithelial stem cell populations

During postembryonic development, Wnt/β-Catenin signaling frequently promotes differentiation of epithelial stem and progenitor populations, including the gut, hair follicle, hippocampus, and the SVZ but also functions to promote stem cell renewal and expansion of gut, hippocampus, and SVZ stem cell populations (Andreu et al., 2008; Choe and Pleasure, 2012; Fevr et al., 2007; Krausova and Korinek, 2014; Lie et al., 2005; Lien et al., 2014; Lowry et al., 2005; Piccin and Morshead, 2011; Pinto et al., 2003; Qu et al., 2010; Varela-Nallar and Inestrosa, 2013; Wang et al., 2011b; Zhou et al., 2004)

Wnt/β-Catenin signaling has long been thought to drive the division and maintenance of stem cells in the intestine. The intestinal epithelium is renewed continually, from a population of stem cells that reside in the base of 'crypts' and produce differentiating cells that are slowly shed from the tips of 'villi'(Fevr et al., 2007). Early experiments demonstrated that when transcriptional activator of Wnt/β-Catenin signaling *tcf4* is deleted, crypts lose undifferentiated stem cells. Additionally, knocking down Wnt/β-Catenin signaling by deletion of β-Catenin or overexpression of Wnt/β-Catenin inhibitor *dkk1* were demonstrated to result in the loss of intestinal crypts (Fevr et al., 2007; Ireland et al., 2004; Pinto et al., 2003). Conversely, activation of Wnt/β-Catenin

signaling by the addition of R-spondin increases the number of Lgr5+ stem cells, indicating that Wnt/β-Catenin signaling functions to renew or expand stem cells in the colon (Kim et al., 2005; Krausova and Korinek, 2014). Famously, the critical β-Catenin destruction complex member *adenomatous polyposis coli (apc*) is found to be mutated in many patients, leading to up-regulated Wnt/β-Catenin signaling in colon tumors, and furthermore, Wnt/β-Catenin activation has been shown to be the driving force behind the majority of intestinal tumors (Albuquerque et al., 2002; Fodde, 2002). Subsequent to stem cell maintenance and division, Wnt signaling is used to promote the differentiation specifically of paneth cells in intestine (Andreu et al., 2008). It is thus clear that Wnt signaling is used iteratively in the gut to both regulate maintenance and expansion of stem cells followed by the promotion of paneth cell fate in differentiating cells.

The mature hair follicle is an organ that undergoes cycles of proliferation and quiescence, either generating a hair fiber or maintaining stem cells, respectively. High levels of BMP signaling drive expression of NFATc1 in multipotent bulge stem cells and functions to keep these cells quiescent. As BMP levels are diminished, stem cells are activated and enter the cell cycle (Plikus et al., 2008) as cell cycle effector gene cdk4 is de-repressed following loss of NFATc1 expression (Horsley et al., 2008). Subsequent to the activation of bulge stem cells by the withdrawal of BMP/NFATc1 signals, Wnt signaling functions to promote differentiation of progeny cells eventually generating a mature hair fiber. Notably, β-catenin is completely dispensible for the proliferation of hair follicle stem cells, but is necessary to activate genes that promoting hair follicle fate and suppressing sebocyte fate determination (Lien et al., 2014). Therefore in this epithelial stem cell population, Wnt/β-Catenin signaling promotes differentiation and

does not activate or maintain stem cells, demonstrating the context-dependent nature of Wnt/β-Catenin regulation of epithelial stem cells.

1.6.3. Wnt/β-Catenin signaling in nervous system stem cell populations

It is well established that Wnt/β-Catenin signaling is necessary to promote differentiation of neural precursor cells in cortical development (Choe and Pleasure, 2012; Munji et al., 2011), midbrain dopaminergic differentiation (Castelo-Branco et al., 2003), and during hippocampal homeostatic neurogenesis (Kuwabara et al., 2009; Seib et al., 2013). In addition to the well-conserved role of Wnt/β-Catenin signaling in neural differentiation, Wnt/β-Catenin signaling also functions specifically in the neural stem cells in the hippocampus and the SVZ where it acts as a mitogen.

In the dentate gyrus of the hippocampus, neural stem cells are RG cells. In this neurogenic zone, activation of Wnt/β-Catenin signaling leads to increased numbers of RG cells as well as increased numbers of newborn neurons (Lie et al., 2005). Others have demonstrated that when the Wnt/β-Catenin inhibitor *dkk1* is deleted, new granule neurons possess increased dendritic complexity (Seib et al., 2013), highlighting Wnt/β-Catenin signaling's role during multiple stages of the neurogenic process. The deletion of another secreted Wnt/β-Catenin inhibitor, *sfrp3*, from dentate granule cells in the hippocampus causes quiescent RG cells to become activated and generate increased numbers of new dentate granule neurons which go on to form complex arbors (Jang et al., 2013). Therefore, Wnt/β-Catenin signaling coordinates multiple steps in hippocampal neurogenesis and suggests that the homeostatic production of neurons is tightly regulated

by feedback inhibition from progeny cells that express inhibitors of Wnt/β-Catenin signaling, subsequently attenuating RG activation.

Wnt/β-Catenin also acts at multiple stages of the neurogenic process in the SVZ of the lateral ventricles, although the SVZ is less characterized than the SGZ. It was initially shown that activation of Wnt/β-Catenin signaling in the CNS, either through germ-line mutation of *apc* (Chenn and Walsh, 2002) or by conditional activation of β-Catenin using (Zechner et al., 2003) the *brn4* promoter to drive expression in early neuroepithelial cells throughout the nervous system, resulted in a large expansion of neural progenitor cells and attenuated differentiation of neurons. Conditional deletion of *beta-catenin* in the *brn4* population resulted in the loss of neuroepithelial cells, demonstrating that prior to the development of astrocytic B-cells in the SVZ, neuroepithelial cells depend on Wnt/β-Catenin signaling for their maintenance and activation leads to expansion of this cell type at the expense of neural differentiation. Subsequently, isolated B-cells were shown to depend on Wnt/β-Catenin signaling to generate new stem cells as well as neurons, astrocytes, and oligodendrocytes in-vitro (Kalani et al., 2008), and Wnt/β-Catenin signaling was shown to be critical for the proliferation of Mash1+ transit amplifying cells in the SVZ (Adachi et al., 2007). Finally, it has been shown that Wnt/β-Catenin signaling is not found in stem cells in conditions where they are dividing asymmetrically and up-regulated when stem cells are dividing symmetrically, for example during regenerative responses. Furthermore Wnt/β-Catenin perturbations demonstrated that Wnt/β-Catenin signaling is necessary for stem cell symmetric division in-vitro and in-vivo (Piccin and Morshead, 2011). Therefore, Wnt/β-Catenin signaling is clearly involved in the regulation of the stem cell pool both by

promoting symmetric stem cell division but also subsequently promoting differentiation in mash1+ transit amplifying cells.

Taken together, these data implicate Wnt/β-catenin signaling functions in the stem/progenitor populations in epithelial tissues including the nervous system's germinal zones to promote proliferation, in addition to the documented roles of Wnt/β-Catenin signaling in promoting differentiation of neurons. In Chapter 3, we have combined reagents to genetically label RGs in the hypothalamus and observe their potential during normal development and during regenerative responses with reagents to perturb Wnt signaling and determine if RG cells themselves require Wnt signaling to support neurogenesis and regeneration in-vivo.

1.7. Modes of stem cell division

To protect themselves from damage associated with a high mitotic rate, during normal homeostatic proliferation and during a response to injury, it has been long thought that stem cells cycle slowly, dividing asymmetrically to self-renew themselves while their progeny differentiate or cycle quickly as transit amplifying cells to generate large numbers of cells that can differentiate to ensure tissue homeostasis or repair injury (Potten, 1974) (Potten & Loeffler, 1990). Asymmetric division is used by radial glia in the hippocampus and developing cortex and by B cells in the SVZ (Bultje et al., 2009), as well as by radial glia in the zebrafish telencephalon (Dong et al., 2012) to self-renew and generate neurons, and used by Mueller glial cells during neural regeneration (Nagashima et al., 2013). However, new data, from epidermis to germ line stem cells, to the gut

(Jones et al., 2007) has generated a model whereby stem cell populations are renewed by stem cells that divide symmetrically yet choose to differentiate or self-renew stochastically (Fig. 1.4.) (Klein and Simons, 2011; Krausova and Korinek, 2014; Morrison and Kimble, 2006). It is possible that stem or progenitor populations can switch between these two modes of division, for example to repair an injury or during development when they need to expand their population to generate large brain structures. Interestingly, in the SZV, Wnt/β-Catenin signaling is necessary and sufficient for the symmetric expansive divisions of stem cells (Piccin and Morshead, 2011), but this population homeostatically divides asymmetrically to generate olfactory bulb neurons while self-renewing. In addition, embryonic neuroepithelial cells are greatly expanded if Wnt/β-Catenin is activated by mutation of *apc*, indicating that Wnt/β-Catenin signaling up-regulation leads to symmetric expansion of these early neural progenitor cells (Chenn and Walsh, 2002). The regulation of symmetric divisions of stem cell populations in-vivo are poorly understood, but are of great relevance. We have been able to test the Wnt/β-Catenin dependence of symmetric radial glial divisions using a partial ablation paradigm

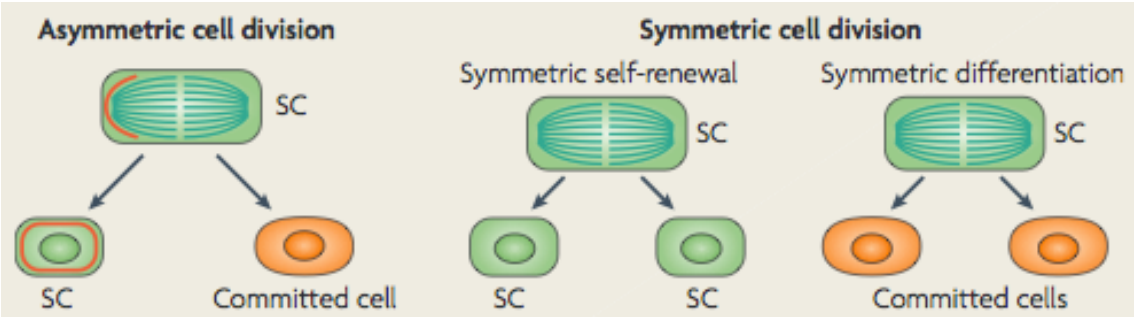

Figure 1.4. Models of stem cell division schemes. Stem cells can divide asymmetrically to generate a self-renewed stem cell and a differentiating cell, or can divide symmetrically to generate two equivalent cells which both can either self renew as stem cells or both differentiate. These are two methods stem cells can use to renew their population- at the individual cell level or at the population level (Blanpain and Fuchs 2009, used with permission).

in the zebrafish hypothalamus and investigate the requirement of Wnt signaling in this regenerative response.

1.8. Regeneration in the CNS

The zebrafish are able to support regeneration in the majority of brain regions studied. Radial glial cells support this regenerative response in the spinal cord (Briona and Dorsky, 2014) and telencephalon (Kroehne et al., 2011; Kyritsis et al., 2012), while Mueller glial cells, a radial glial subtype, support neural regeneration in the retina(Lenkowski et al., 2013). Interestingly, zebrafish do not possess radial-shaped astrocytes (Grupp et al., 2010) which could functionally contribute to the remarkable proliferative and regenerative nature of the CNS. Astrocytic activation contributes greatly to glial scarring by secreting glycoproteins that largely inhibit the proliferation and axonal regrowth critical for neural regeneration. The mammalian CNS supports neural regeneration of dentate granule cells in the hippocampus (Ogita et al., 2005), hypothalamic neurons (Cao et al., 2002), and retina amacrine neurons (Karl et al., 2008). Interestingly, in models of stroke leading to death of cortical and striatal neurons, SVZ-generated neuroblasts alter their migratory paths from the olfactory bulb to the injury site, yet fail to repair the tissue (Kahle and Bix, 2013; Yamashita et al., 2006), likely due to the presence of an inhibitory environment.

1.9. Zebrafish hypothalamus as a model of adult neurogenesis

Our lab has previously demonstrated that Wnt/β-Catenin signaling is necessary for the differentiation of neurons in the zebrafish posterior recess (PR) of the

hypothalamus; however, we were surprised by the striking phenotype of the Wnt/β-Catenin signaling loss of function mutant, *lef1*. These animals display a smaller PR that is progressively more dramatic during postembryonic growth. As well as possessing neural progenitor cells that are stuck at the Sox3+ state, failing to mature into HuCD+ neurons, the *lef1* mutant hypothalamus has fewer cells in general yet does generate RG cells. This phenotype implies that *lef1* is critical for proper stem cell function. If *lef1* were only necessary for proper differentiation, then the PR of the lef1 mutants would possess the correct number of cells, yet would all be stuck at the Sox3+ neural progenitor state. We therefore hypothesized that Wnt/β-Catenin signaling is necessary for the activation and proliferation of Radial glia cells in addition to its subsequent role in promoting hypothalamic differentiation.

1.10. Summary

My thesis has made significant progress in the study of the neural progenitor cells and the potential regulation of these remarkable cells by Wnt/β-Catenin signaling. Prior to this analysis, there have been no rigorous characterizations of the necessity and sufficiency of Wnt/β-Catenin signaling in postembryonically labeled RG populations during homeostatic proliferation and regeneration (Fig. 1.5.). A parsimonious hypothesis synthesizing the documented roles of Wnt/β-Catenin signaling in the regulation of epithelial stem cells, especially neural stem cell populations, suggests that Wnt/β-Catenin signaling functions to activate radial glial cells to enter the cell cycle, and subsequently, Wnt/β-Catenin functions in this population to promote differentiation.

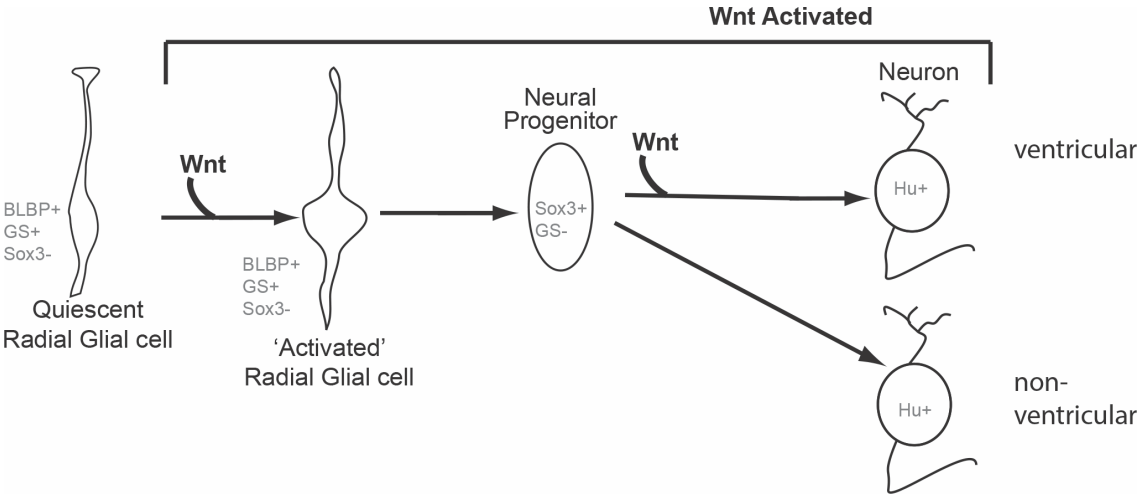

Figure 1.5. A model of Wnt/β-Catenin regulation of radial glial cell activation. We hypothesize that Wnt/β-Catenin signaling 'activates' radial glial cells to leave quiescence and divide to either generate new neurons (pictured) in development or regeneration or to divide symmetrically to generate two new radial glial cells during normal homeostasis or during regeneration (not pictured). Subsequent to the utilization of Wnt/β-Catenin signaling in radial glial progenitor cells, Wnt/β-Catenin signaling functions to generate ventricular HuC/D+ neurons.

First, I have performed an expression analysis of the Wnt/β-Catenin gene family of ligands in the CNS to determine which Wnt/β-Catenin ligands drive Wnt/β-Catenin signaling in the hypothalamus and other germinal zones in the zebrafish CNS. Next, I have used Cre-mediated lineage tracing to show that hypothalamic RG cells generate multiple classes of neurons and are maintained in the long term. I have shown that Wnt/β-Catenin signaling is not required for the proliferation, renewal, or maintenance of RG cells in normal homeostasis, but is only necessary for the generation of a subset of ventricular-located neurons. Additionally, I have demonstrated that RG cells in the posterior recess support regeneration of their own population as well as dopaminergic neurons. I have subsequently shown that RG regenerative proliferation is not dependent

on Wnt/β-Catenin signaling. Taken together, this work demonstrates that neural progenitor cells in different brain regions are heterogeneously regulated to proliferate, and Wnt/β-Catenin signaling is not necessary for the proliferation and expansion of RG cells in the posterior recess. Finally, we have determined that it is necessary for Wnt/β-Catenin signaling to be kept low in RG cells for them to be maintained or self-renew.

1.11. References

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CHAPTER 2

IDENTIFICATION OF WNT GENES EXPRESSED IN NEURAL PROGENITOR ZONES DURING ZEBRAFISH BRAIN DEVELOPMENT

2.1. Abstract

Wnt signaling regulates multiple aspects of vertebrate central nervous system (CNS) development, including neurogenesis. However, vertebrate genomes can contain up to 25 *Wnt* genes, the functions of which are poorly characterized partly due to redundancy in their expression. To identify candidate *Wnt* genes as candidate mediators of pathway activity in specific brain progenitor zones, we have performed a comprehensive expression analysis at three different stages during zebrafish development. Antisense RNA probes for 21 *Wnt* genes were generated from existing and newly synthesized cDNA clones and used for in-situ hybridization on whole embryos and dissected brains. As in other species, we found that *Wnt* expression patterns in the embryonic zebrafish CNS are complex and often redundant. We observed that progenitor zones in the telencephalon, dorsal diencephalon, hypothalamus, midbrain, midbrain-hindbrain boundary, cerebellum, and retina all express multiple *Wnt* genes. Our data identify 12 specific ligands that can now be tested using loss-of-function approaches.

2.2. Introduction

Wnt/β-Catenin signaling is known to act in multiple ways to regulate vertebrate central nervous system (CNS) development, including as a mitogen (Megason and McMahon, 2002), and in neural specification and differentiation(Agathocleous et al., 2009; Munji et al., 2011; Wang et al., 2012). The pathway also functions in post-embryonic neurogenesis, to promote the differentiation of neural progenitor cells in the dentate gyrus of the hippocampus (Lie et al., 2005), the rostral migratory stream (Imura et al., 2010)**,** and the hypothalamus(Wang et al., 2012). However, with a few exceptions

such as *wnt7a* in the dentate gyrus (Qu et al., 2010), it has been difficult to link functions in defined neural progenitor populations to specific Wnt ligands, possibly due to extensive redundancy within the Wnt family (Farin et al., 2012; Ikeya et al., 1997). In addition, different Wnt ligands can activate multiple downstream pathways in the same tissue, such as in the zebrafish fin where *wnt10a* and *wnt5b* are both required for regeneration through ß-catenin-dependent and independent signaling, respectively (Stoick-Cooper et al., 2007). It is therefore important to first precisely identify the specific Wnt ligands expressed in each neural progenitor population in order to test their function in neurogenesis.

Our laboratory is interested in the role of Wnt/ß-catenin signaling in hypothalamic neurogenesis, where we have previously shown a requirement for Lef1-mediated transcription in progenitor differentiation(Wang et al., 2012). While we have identified one candidate ligand (*wnt8b*) in this region, knockout of this gene in mouse does not produce significant defects in brain development (Fotaki et al., 2010). We thus decided to systematically examine the expression of the entire *Wnt* gene family in the developing zebrafish CNS in order to identify other candidates that may regulate hypothalamic neurogenesis. While many of these genes have been previously reported to have expression in specific brain regions (zfin.org), others have not been characterized and no single study has compared all the patterns at multiple stages.

For this work, we examined the expression of 21 *Wnt* genes that either had known brain expression or were previously unexamined. While a comprehensive analysis of *Wnt* gene expression during early developmental stages has been previously performed (Lu et al., 2011), we carried out our experiments at 24, 48, and 72 hours post-fertilization (hpf),

to cover both embryonic and postembryonic CNS progenitor populations. At 48 and 72 hpf, we specifically focused on known progenitor zones, including the telecephalic pallium/subpallium (Marz et al., 2010), the dorsal diencephalon (epithalamus and thalamus) (Grandel et al., 2006), the ventral diencephalon (hypothalamus)(Wang et al., 2012), the midbrain (Ito et al., 2010), the midbrain/hindbrain boundary (Leucht et al., 2008), the cerebellum (Kaslin et al., 2009), and the ciliary marginal zone (CMZ) of the retina (Wehman et al., 2005). Ultimately, we were able to identify 12 genes with specific brain expression at all stages, most of which were localized to progenitor zones, and we found 3 genes (*wnt8b, wnt11r*, and *wnt16*) expressed in the hypothalamic posterior ventricular recess. Our results highlight the redundancy of Wnt ligand expression during zebrafish development, and lay the foundation for future functional analysis of Wnt signaling throughout the CNS.

2.3. Results

2.3.1. Expression in the 24 hpf brain

We generated antisense probes for 21 zebrafish *Wnt* genes either from previously published DNA templates, or by RT-PCR amplification and subcloning (Table 2.1). The only known *Wnt* genes that we did not examine were *wnt7ab* and *wnt8a* due to their reported lack of CNS expression after somitogenesis (Beretta et al., 2011; Kelly et al., 1995), and *wnt6a* and *wnt6b* due to their annotation after the initiation of this project. At 24 hpf, much of the CNS is still rapidly proliferating and undergoing neurogenesis, and we observed expression of multiple *Wnt* genes expression throughout the brain (Fig. 2.1, Table 2.2). We found only two genes (*wnt7ba* and *wnt8b*) expressed in the telencephalon

Table 2.1. Sources of published in-situ probe templates or primers used to amplify cDNA.

Gene	Reference or primers
wnt1	(Dorsky et al., 1998)
wnt2	(Veien et al., 2008)
wnt2ba	(Veien et al., 2008)
wnt2bb	(Ober et al., 2006)
wnt3	(Clements et al., 2009)
wnt3a	(Dorsky et al., 1998)
wnt4a	(Ungar et al., 1995)
wnt4b	F: TGTATTTGATGTGTCGGCCA R: ACGCAGACACTTTGCCTTTT
wnt5a	F: ATGATGCTGCTGAAGCTGAAGT
wnt5b	F: GGAAGGATGGATGTGAGAATGAA R:
wnt7aa	F: ATGAGCAGGAAAACGCGC R:
wnt7ba	(Carl et al., 2007)
wnt7bb	(Carl et al., 2007)
wnt8b	(Kelly et al., 1995)
wnt9a	F: GGAGAAGAAGCAGCGCAGAA R:
wnt9b	F: GGGATTTCAACACGGACAGATAG R:
wnt10a	F: ATGAGCTCTCACGACATCAGTTG R:
wnt10b	F: GTTCGACGCAATGGAGTTACC R:
wnt11	(Ulrich et al., 2003)
wnt11r	(Jing et al., 2009)
wnt16	Gift from Gilbert Weidinger

(Fig. 2.1 L',N'). Eleven genes were expressed in the diencephalon, including seven in the epithalamus (Fig. 2.1A',E',F',G',K',L'M',N'), one (*wnt4b*) in the thalamus (Fig. 2.1H'), and three (*wnt 8b, wnt11r,* and *wnt16*) with expression in the hypothalamus (Fig. 2.1N',T',U'). Ten genes were expressed in the midbrain, including six in the dorsal (tectum) region (Fig. 2.1A',E',F',K',M',R'), four at the midbrain-hindbrain boundary (Fig. 2.1A',E,N',R'), and three in the ventral (tegmentum) region (Fig. 2.1G',H',T'). Four genes were expressed in the rostral hindbrain (cerebellum) (Fig. 1A',E',K',R'). We also observed one gene (*wnt4b*) with expression in the floor plate of the hindbrain and spinal cord (Fig. 2.1H). Nine other genes showed low-level ubiquitous or nonspecific expression throughout the

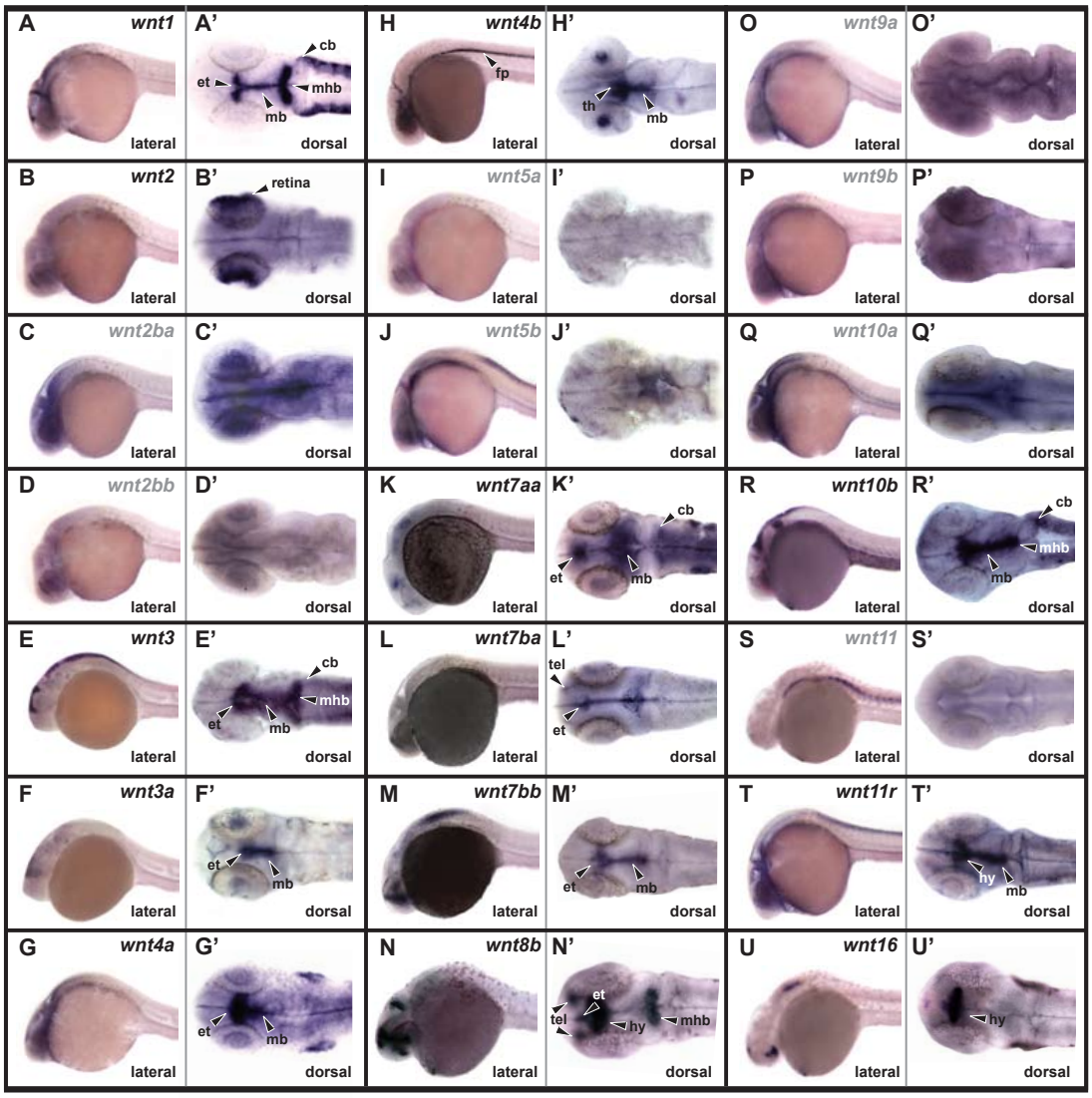

Figure 2.1. *Wnt* gene expression at 24 hpf. Lateral views of whole embryos are shown in left panels, and dorsal views of dissected brains are shown in right panels. Genes with nonspecific brain expression are indicated with grey text. tel, telencephalon; et, epithalamus; th, thalamus; hy, hypothalamus; mb, midbrain; mhb, midbrain-hindbrain boundary; cb, cerebellum; fp, floor plate.

Table 2.2. *Wnt* expression in the developing zebrafish brain. p, pallium; sp, subpallium; et, epithalamus; th, thalamus; pr, posterior recess; d, dorsal; v, ventral; m, midbrain/hindbrain boundary; cb, cerebellum.

	Telencephalon			Dorsal Diencephalon			Hypothalamus			Midbrain			Cerebellum			Retina (CMZ)	
	24 hpf	48 hpf	72 hpf	24 hpf	48 hpf	72 hpf	24 hpf	48 hpf	72 hpf	24 hpf	48 hpf	72 hpf	24 hpf	48 hpf	72 hpf	24 hpf	48 hpf
wnt1				et	et	et				dm	dm	dm					
wnt2																	
wnt2ba																	
wnt2bb																	
wnt3				et	et	et				dm	dm	dm					
wnt3a				et	et th	et				d	d	d					
wnt4a				et	et	et				v	v	v					
wnt4b				th	th					v	v	v					
wnt5a																	
wnt5b																	
wnt7aa		p	p	et	et	et				d	d	d					
wnt7ba		p	p	et	et	et					d	d					
wnt7bb		p		et	et	et				d	d						
wnt8b		sp	sp	et				pr	pr	m							
wnt9a																	
wnt9b																	
wnt10a																	
wnt10b					et	et				dm	dm	dm					
wnt11																	
wnt11r								pr	pr	v							
wnt16					th	th		pr	pr								

brain (Fig. 2.1B-D',I-J',O-Q',S,S'); however, we did observe specific expression of one gene (*wnt2*) in the retinal margin (Fig. 2.1B').

2.3.2. Expression in the 48 hpf brain

By 48 hpf the CNS has largely completed morphogenesis, and several proliferative neurogenic zones are retained near the ventricles (Mueller and Wullimann, 2003). At this stage and beyond, we only analyzed expression patterns of the 12 genes that were specifically expressed at 24 hpf (Fig. 2.2, Table 2.2). In the telencephalon, expression of *wnt7aa, wnt7ba,* and *wnt7bb* was localized to the dorsal (pallial) region and *wnt8b* expression was localized to the ventral (subpallial) region (Fig. 2.2F-I'). In the dorsal diencephalon, eight genes were expressed in the epithalamus (Fig. 2.2A-D',F-H',J,J'), and three genes (*wnt3a, wnt4b* and *wnt16*) were expressed in the thalamus (Fig. 2.2B,B',E,E',L,L'). At 48 hpf, the third ventricle of the hypothalamus has elaborated into bilateral recesses(Wang et al., 2012), and we observed specific expression of *wnt8b, wnt11r* and *wnt16* in the posterior recess as viewed from the ventral brain surface (Fig. 2.2I,I',K-L'). Nine genes were expressed in the midbrain (Fig. 2.2A-H',J,J'), including three (*wnt1, wnt3,* and *wnt10b*) in the midbrain-hindbrain boundary (Fig. 2.2A-B',J,J'), and two (*wnt4a, wnt4b*) in the ventral midline as well as the hindbrain floor plate (Fig. 2.2D-E'). The same four genes were expressed in the cerebellum as at 24hpf, with the addition of *wnt7bb* (Fig. 2.2A-B',F-G',J,J'). Finally, we observed expression of four genes in the ciliary marginal zone (CMZ) of the retina (Fig. 2.3 and Table 2.2), which is the region containing neural progenitors (Wehman et al., 2005).

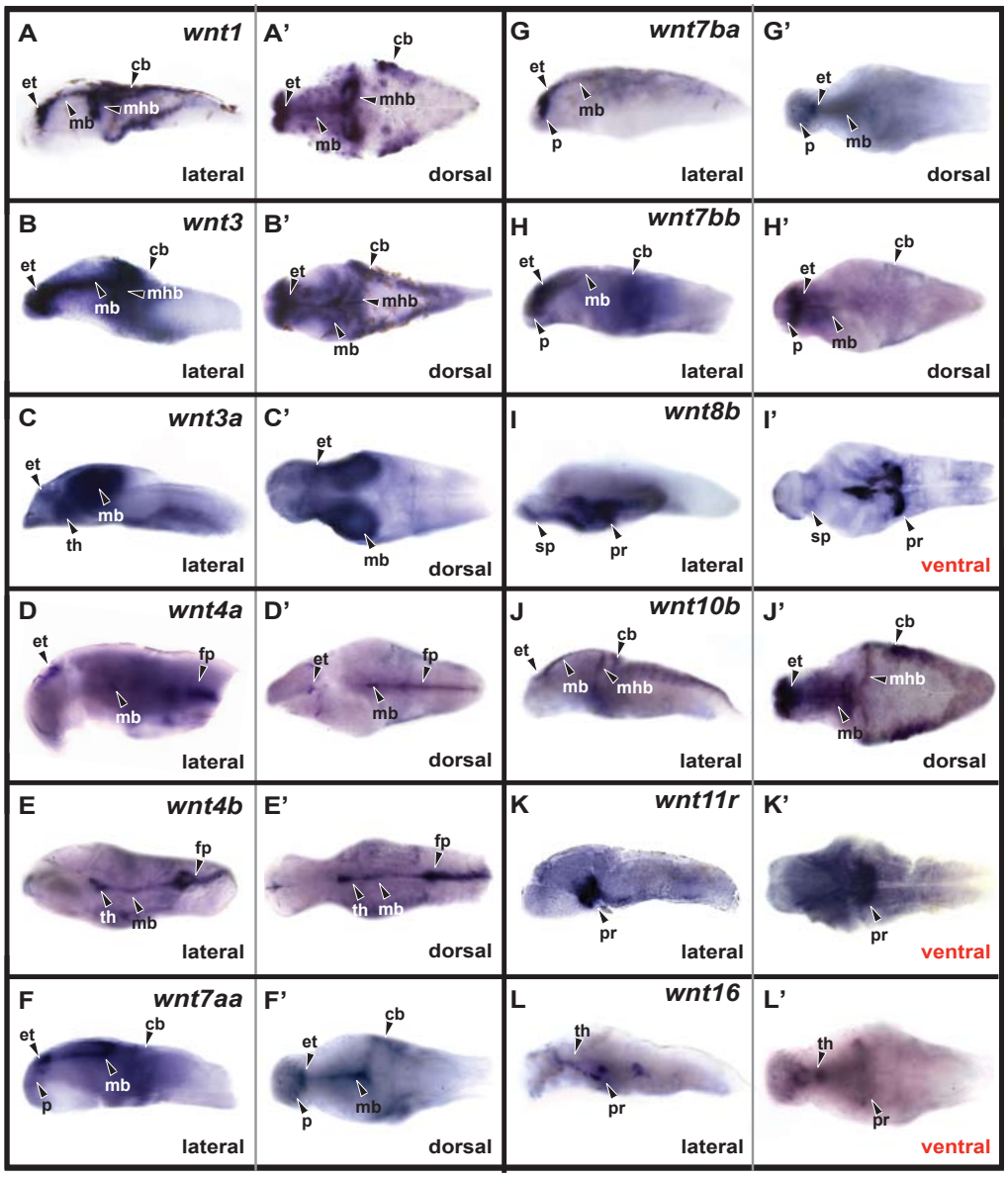

Figure 2.2. *Wnt* gene expression at 48 hpf. Lateral views of dissected brains are shown in left panels, and dorsal or ventral views of dissected brains are shown in right panels. p, pallium; sp, subpallium; et, epithalamus; th, thalamus; pr, posterior recess; mb, midbrain; mhb, midbrain-hindbrain boundary; cb, cerebellum; fp, floor plate.

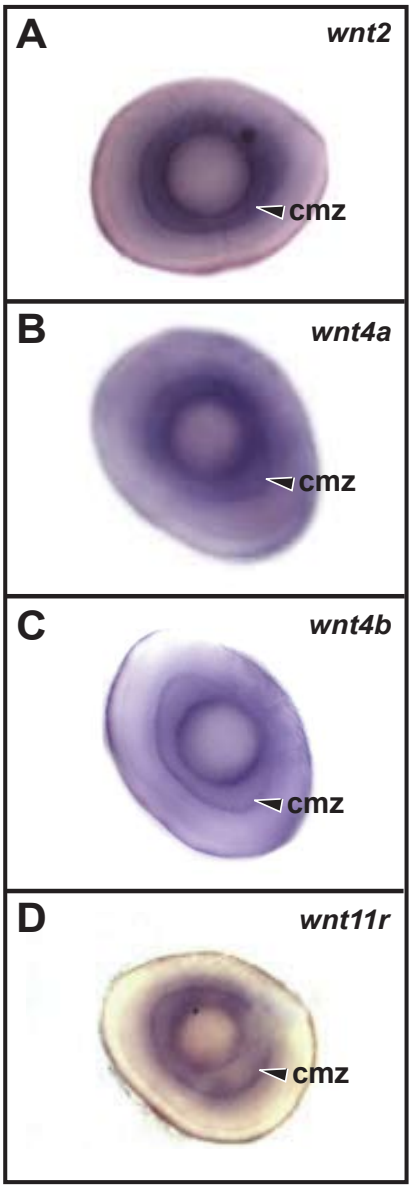

Figure 2.3. *Wnt* gene expression in the retina at 48 hpf. Dissected eyes are shown in all panels. cmz, ciliary marginal zone.

2.3.3. Expression in the 72 hpf brain

Due to the larger brain size at 72 hpf, we were only able to characterize specific expression patterns using dorsal or ventral whole-mount views (Fig. 2.4 and Table 2.2). We found that *wnt7aa* and *wnt7ba* were expressed in the pallium (Fig. 2.4F,G), and *wnt8b* was still expressed in the subpallium (Fig. 2.4I). The same eight genes were expressed in the epithalamus as at 48 hpf (Fig. 2.4 A-D,F-H,J), and *wnt16* continued to be expressed in the thalamus (Fig. 2.4L). Similarly, *wnt8b, wnt11r,* and *wnt16* continued to be expressed in the hypothalamic posterior recess (Fig. 2.4I,K,L), and eight genes were expressed in the midbrain and midbrain-hindbrain boundary, (Fig. 2.4A-G,J). Expression of *wnt3, wnt7aa,* and *wnt10b* was observed in the cerebellum (Fig. 2.4B,F,J) and expression of *wnt4a* and *wnt4b* was maintained in the floor plate (Fig. 2.4D,E).

2.4. Discussion

Our data clearly demonstrate the coincident expression of multiple *Wnt* genes in progenitor zones of the developing CNS (Table 2.1), and support previous observations of gene redundancy. Some of the overlapping gene expression that we observed could be explained by closely related orthologs arising from the teleost genome duplication (Postlethwait et al., 1998), such as in the cases of *wnt4a/b* and *wnt7ba/bb*. However, other related genes that are not teleost-specific duplicates, such as *wnt2/2b, wnt3/3a, wnt5a/b, wnt10a/b,* and *wnt11/11r*, have clearly different expression patterns indicating that their transcriptional regulation has likely diverged considerably. Investigations using double in-situ hybridization and higher resolution analysis of specific CNS tissues can

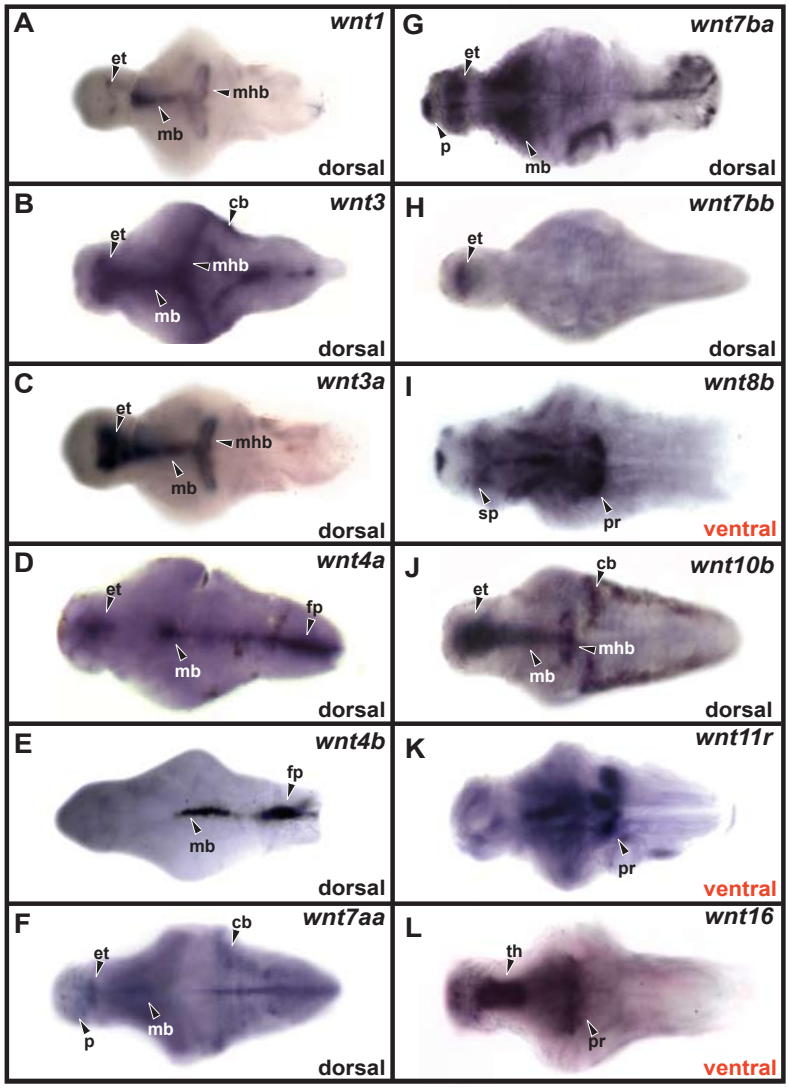

Figure 2.4. *Wnt* gene expression at 72 hpf. Dorsal or ventral views of dissected brains are shown in all panels. p, pallium; sp, subpallium; et, epithalamus; th, thalamus; pr, posterior recess; mb, midbrain; mhb, midbrain-hindbrain boundary; cb, cerebellum; fp, floor plate.

also be used to identify more subtle differences between grossly overlapping gene expression patterns.

Expression of *Wnt* genes encoding ligands that signal through ß-catenin-independent pathways also precludes simple predictions of redundant function. Importantly, both ß-catenin-dependent and -independent pathways play roles in stem cell

maintenance and differentiation (Clements et al., 2011; Katoh, 2008), suggesting that postembryonic neurogenesis may be regulated by multiple Wnt outputs in the same tissue.

The data presented here will be useful for the identification of candidate Wnt ligands that could mediate specific processes in CNS development, as demonstrated by the distinct subsets of *Wnt* genes expressed in consistent regional domains. For example, *wnt1, wnt3, wnt3a, wnt7aa,* and *wnt10b* are all localized to the roof plate of the diencephalon, midbrain, and cerebellum. In contrast, *wnt4a* and *wnt4b* show consistent expression in floor plate structures, and *wnt8b, wnt11r,* and *wnt16* are expressed in the hypothalamus. In addition, our analysis offers a starting point for further studies investigating the functions of Wnt ligands in developmental and postembryonic neurogenesis. The identification of three ligands expressed in the hypothalamic posterior recess provides specific targets for manipulating Wnt pathway activity in a defined model of neural progenitor maintenance and differentiation, especially as mutant alleles are available for all three genes (Gordon et al., 2012). With the recent development of effective gene targeting methods in zebrafish (Hwang et al., 2013), it will be possible to produce animals carrying null alleles for all the potential *Wnt* genes expressed in or near a given CNS tissue or cell population.

2.5. Materials and methods

2.5.1. Zebrafish embryo maintenance

All fish were used and handled in accordance with University of Utah IACUC guidelines. Fertilized wild-type (AB*) zebrafish embryos were and staged according to

Kimmel et al. (Kimmel et al., 1995), and raised until 24, 48, and 72 hours post-fertilization (hpf). Embryos were fixed overnight at 4°C in 4% paraformaldehyde with 5% sucrose. Brains were manually dissected for in-situ hybridization at 48 and 72 hpf.

2.5.2. Cloning of zebrafish Wnt genes

The Ensembl *Danio rerio* genome database was used to identify genomic loci for all unpublished genes. Primers were designed to amplify ~500bp cDNA fragments for each gene (Table 2.1), and RT-PCR was performed on total RNA extracted from 24 hpf embryos using a Superscript II kit (Invitrogen). Amplicons were then subcloned into PCRII-TOPO (Invitrogen), and sequenced to verify gene identity as well as to confirm orientation for generation of antisense RNA probes.

2.5.3. In-situ hybridization

Antisense riboprobes were synthesized and in-situ hybridization was performed as previously described (Thisse and Thisse, 2008), with the addition of 5% dextran sulfate (Sigma-Aldrich D8906) to the hybridization buffer. Posthybridization washes were carried out using a Biolane HTI in-situ machine (Huller and Huttner AG). After staining, embryos were stored in MeOH and cleared in 70% glycerol for dissection and imaging. Images were acquired using an Olympus BX51WI compound microscope and an Olympus Microfire camera. Digital images were cropped and aligned using Adobe Photoshop.

2.6. Acknowledgements

We thank Wilson Clements, Elke Ober, Matthias Carl, Gilbert Weidinger and Arne Levken for generously sharing plasmid templates for in situ probes.

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CHAPTER 3

MAINTENANCE OF RADIAL GLIA AS SELF-RENEWING NEURAL PROGENITORS REQUIRES THE ABSENCE OF WNT SIGNALING

3.1. Abstract

The vertebrate hypothalamus contains persistent populations of radial glia that have been proposed to function as neural progenitors. In zebrafish, a high level of postembryonic hypothalamic neurogenesis has been observed, but the role of radial glia in generating these new neurons is unknown. We have used inducible Cre-mediated lineage labeling to show that hypothalamic radial glia undergo self-renewal and generate multiple neuronal subtypes. While Wnt/ß-catenin signaling has been shown to regulate the maintenance and self-renewal of other stem and progenitor cell populations, we find that these processes instead require the absence of Wnt activity in hypothalamic radial glia, and Wnt signaling is only required for the differentiation of specific neuronal progeny. We also show that hypothalamic radial glia respond to genetic ablation of themselves and their progeny by increasing their proliferation, and that this process similarly does not require Wnt/ß-catenin signaling. Hypothalamic radial glia thus exhibit characteristics of a neural stem cell population, and our data support the idea that Wnt signaling may not be homogeneous in all stem cells.

3.2. Introduction

The postembryonic teleost brain is highly proliferative and highly regenerative (Kizil et al., 2012b; Kroehne et al., 2011), due to the presence of radial glia that persist throughout the CNS and generate neurons (Kriegstein and Alvarez-Buylla, 2009). We previously characterized a population of neural progenitors in the postembryonic zebrafish hypothalamus, which produce multiple neuronal subtypes through adulthood (Wang et al., 2012). A similar process also occurs in the mammalian hypothalamus,

where adult neurogenesis contributes to reproductive and feeding behaviors (Cheng, 2013; Lee et al., 2012). However, the underlying stem cell population supporting hypothalamic neurogenesis has not been conclusively identified in any vertebrate species. While radial glia have been proposed to fulfill this role in both zebrafish and mouse (Haan et al., 2013; Lee et al., 2012; Malatesta and Gotz, 2013; Pinto and Gotz, 2007), their capacity for self-renewal and neurogenesis has not been experimentally tested.

In addition, the molecular pathways regulating radial glial neural progenitors in the hypothalamus are poorly understood. Although Notch signaling has been shown to be necessary for telencephalic radial glia to maintain quiescence (Chapouton et al., 2010) and FGF signaling has been shown to be necessary for proliferation of cerebellar and hypothalamic radial glia (Bosco et al., 2013; Kaslin et al., 2009; Robins et al., 2013), the roles of other signals have not been characterized. Our previous work showed that Wnt/β-Catenin signaling is required for postembryonic hypothalamic neurogenesis (Wang et al., 2011a), and along with other studies suggested that pathway activity promotes radial glial differentiation (Choe and Pleasure, 2012; Lee et al., 2006; Varela-Nallar and Inestrosa, 2013; Wang et al., 2011a). In contrast, Wnt/ß-catenin signaling has also been implicated in the self-renewal and amplification of neural stem cells in the mammalian subventricular zone and dentate gyrus, (Qu et al., 2010). The specific function of Wnt activity in hypothalamic radial glia is therefore unclear, leaving an open question as to whether a general role for the pathway exists for all stem cell populations.

Here we take a genetic approach to characterize the response and regulation of hypothalamic radial glia during normal homeostatic neurogenesis and regeneration. Our data show that in the postembryonic zebrafish hypothalamus, radial glia are both self-

renewing and multipotent, and exhibit a proliferative response to partial ablation of themselves or their neuronal progeny. In addition, we use multiple perturbations of Wnt/β-catenin signaling to test the necessity and sufficiency of pathway activity for radial glial maintenance, proliferation, and neuronal differentiation. Surprisingly, our data show that Wnt/β-catenin signaling is only necessary for the terminal differentiation of specific neuronal progeny. However, consistent with existing models from studies of non-neural stem cells (Blanpain and Fuchs, 2009; Farin et al., 2012; Lowry et al., 2005), and with observations of radial glia in other brain regions (Wang et al., 2011a), we find that the absence of Wnt/ß-catenin signaling is required to maintain a persistent population of neurogenic radial glia in the hypothalamus.

3.3. Materials and methods

3.3.1. Use of zebrafish

Embryos were obtained from the following zebrafish lines: *Tg(her4.3:EGFP)*^{y83} *(Yeo et al., 2007)*, *Tg(ubi:loxP-eGFP-loxP-mCherry)*^{cz1701} (Mosimann et al., 2011), *Tg(-3her4.1:ERT2-Cre-ERT2)*^{vu298} *(Boniface et al., 2009; Mosimann et al., 2011)), Et(Gal4-VP16,myl7:gfp)*^{zc1066a} (Wang et al., 2012), *Tg(UAS-E1b:NTR-mCherry)*^{jh17} (Davison et al., 2007; Pisharath et al., 2007), *Tg(7xTCF-Xla.Siam:GFP)*^{ia4} (Moro et al., 2012), *Tg(7xTCF-Xla.Siam:nlsmCherry)*^{ia5} (Moro et al., 2012), *Tg(hsp701:dkk1-GFP)*^{w32} (Stoick-Cooper et al., 2007)*, Tg(hsp70l:wnt8a-GFP)*^{w34} *(Weidinger et al., 2005), Tg(th2:GFP-aequorin)*^{zd201} *(McPherson et al., submitted), and Tg(th2:Gal-VP16)*^{zd202} *(McPherson et al., submitted). Embryos were* staged according to (Kimmel et al., 1995). All experiments were approved by the University of Utah Institutional Animal Care and

Use Committee.

Transgenic embryos were identified by GFP fluorescence following heat shock induction of *wnt8* or *dkk1*, or by PCR amplification of trunk tissue for *dkk1* induction in the presence of the *–3.5ubi:loxP-EGFP-loxP-mCherry* reporter using the following primers: *(dkk1* forward*:* tcgactcaaggatcaccaca, *mgfp5* reverse: tccctcaaacttgacttcagc). *lef1* mutant animals were identified by the absence of posterior neuromasts as labeled with DASPEI (McGraw et al., 2011; Wang et al., 2012).

3.3.2. Treatment of embryos and larvae

Cre-mediated recombination was performed by adding 5uM 4-Hydroxytamoxifen (4-OHT) (Sigma, CAS RN 68047-06-3) to embryo medium (E3) from 5 days post-fertilization (dpf) to 6 dpf. Ablations were performed using the addition of 1mM Metronidozole (MTZ) (Fluka 46461 'Vetranal') to E3 from 5-6 dpf. For BrdU labeling, embyros were incubated in 10mM BrdU for 1 day prior to fixation for all experiments. Heat-shock experiments were performed by incubating embryos in 50mL conical tubes in a 39.5C water bath for 20 minutes.

3.3.3. Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde with 5% sucrose overnight at 4C. Brains were then dissected for immunohistochemistry and trunks were placed in PCR tubes for genotyping. Whole brains were washed in water, incubated in 2N HCl for 20 minutes at room temperature, washed, and permeabilized with one unit of Dispase (Gibco 17105-041) for 90 minutes at room temperature. Primary antibodies were all used at

1:500 dilutions and incubated overnight at 4C: mouse anti Glutamine Synthetase (Millipore MAB302), rabbit anti Brain Lipid Binding Protein (Abcam ab32423), rabbit anti DsRed (Clontech 632496), chicken anti GFP (Aves Labs GFP-1020), chicken anti BrdU (Immunology Consultants Laboratory CBDU-65A-Z), rabbit anti 5-HT (ImmunoStar 541016), mouse anti HuC/D (Molecular Probes A21271). Following washes, secondary antibodies (diluted 1:500 with Hoechst 33342) were incubated overnight at 4C. Brains were imaged on a Nikon A1 confocal microscope with a 60X oil objective. The entire posterior recess was imaged using 3uM steps encompassing roughly 40uM total. Cell counting was performed using Nikon software and images were exported to Adobe Photoshop, Adobe Illustrator and ImageJ (NIH) for figure generation.

3.3.4. Statistics

For statistical analysis, Microsoft Excel was used to perform two-tailed equal variance T-tests, and p-values of 0.05 or less were determined to be significant.

3.4. Results

3.4.1. Radial Glia are multipotent neural progenitors in the postembryonic hypothalamus

We previously showed that the *Et(Gal4-VP16,myl7:gfp)*^{zc1066a} enhancer trap line can label radial glia in the zebrafish hypothalamic posterior recess (Wang et al., 2012). We found that at 5 days post-fertilization (dpf) Glutamine Synthetase (GS) (Boniface et al., 2009; Kizil et al., 2012a) and a *her4.3:EGFP* transgene (Yeo, et al., 2007) also label this population heterogeneously (Fig. 3.1A,B). Co-expression analysis showed that the majority of cells express both markers, while others only express *her4.3:EGFP*, or GS

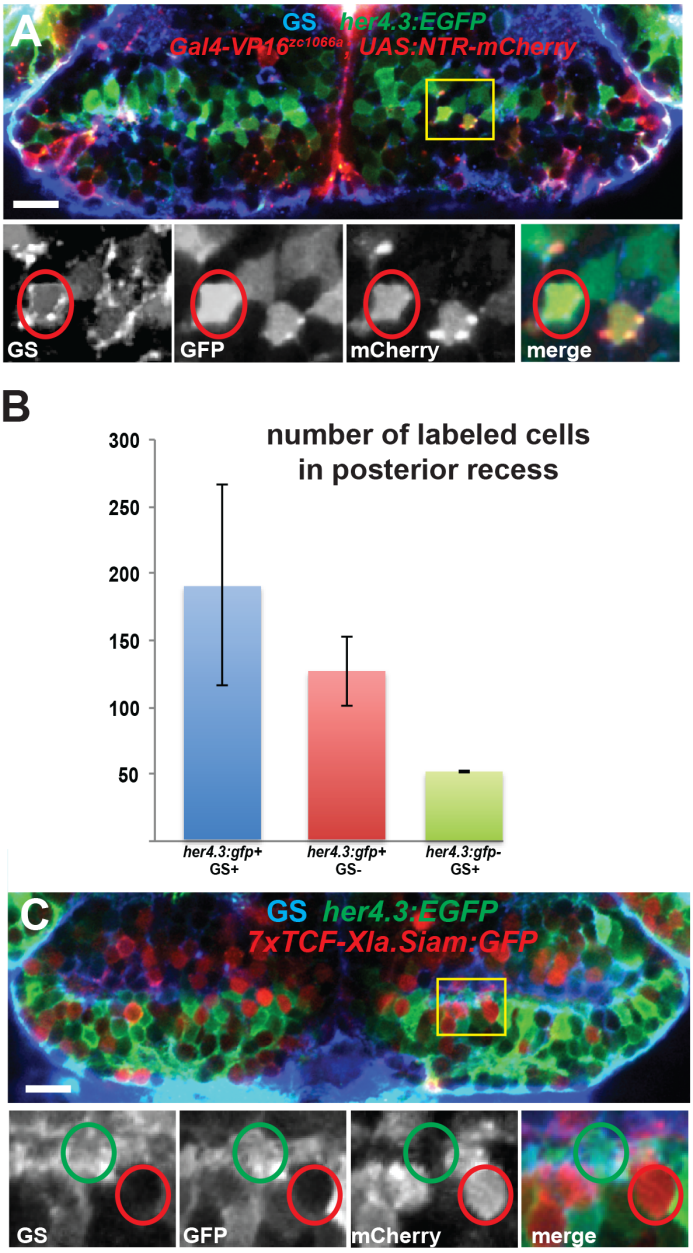


Figure 3.1. Radial glia in the hypothalamic posterior recess are not Wnt-responsive. A) Radial glial cells in the 5 dpf posterior recess can be labeled with anti-Glutamine Synthetase (GS, blue), a *her4.3:EGFP* transgene (green), and a Gal4 enhancer trap line (red). Yellow box indicates region shown in lower panels. A triple labeled cell is indicated by red circles. (B) GS and *her4.3:EGFP* label overlapping subsets of radial glia in the hypothalamus at 5 dpf. Error bars = S.D., N=3 brains. (C) Most radial glia do not express the Wnt reporter transgene *7xTCF-Xla.Siam:GFP*. Yellow box indicates region shown in lower panels. A reporter-negative radial glial cell is indicated by green circles, and a reporter-expressing non-glial cell is indicated by red circles. Images are maximum intensity confocal Z-projections from ventral views of whole-mount brains. Scale bars = 10uM.

(Fig. 3.1B). Consistent with our previous work (Wang et al., 2012), we also found that only an average of 5.5% ± 2.5% (S.E.M., N=11 slices from 2 brains) of GS+ cells expressed the Wnt reporter transgene *7xTCF-Xla.Siam:GFP* (Moro et al., 2012) (Fig. 3.1C).

To determine the lineage of radial glia and their progeny, we took advantage of an existing transgenic line that uses the *her4.3* promoter/enhancer to drive the expression of inducible Cre recombinase (Boniface et al., 2009). By crossing this line to the floxed *ubi:switch* reporter, (Mosimann et al., 2011), we were thus able to permanently label all progeny with mCherry. Following addition of 4-hydroxytamoxifen (4-OHT) from 5-6 dpf, 97% ± 5% of mCherry+ cells (S.D., N=3 brains) were labeled by the radial glial marker GS (Fig. 3.2A). By 6 weeks post-fertilization (wpf), labeled clones were dramatically expanded (Fig. 3.2B) and contained only 8.7% ± 2.6% GS+ cells (S.D., N= 30 slices from 3 brains, Fig. 3.2C). By using markers for differentiated neuronal cell types, we found that at 6 wpf the lineage included neurons labeled by HuC/D (Fig. 3.2D), serotonin (Fig. 3.2E) and a transgenic marker of dopaminergic fate [*Tg(th2:Gal-VP16)*^{zd202}**]** *(McPherson et al., submitted)* (Fig. 3.2F). These data indicate that postembryonic neurons in the zebrafish hypothalamus arise from a radial glial population that can self-renew and generate multiple types of progeny.

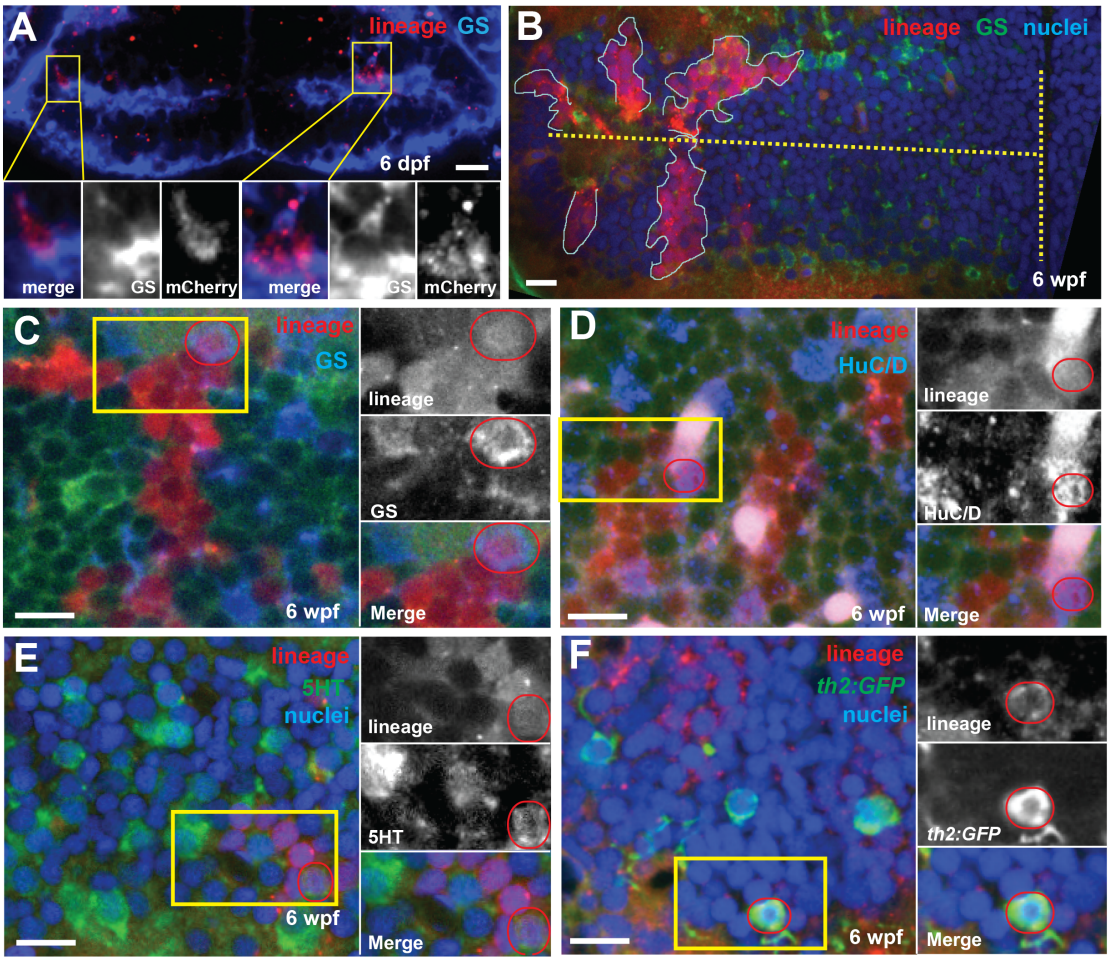


Figure 3.2. Hypothalamic radial glia are self-renewing neural progenitors. (A) Cells expressing *-3her4.1:ERT2-Cre-ERT* and *ubi:loxP-eGFP-loxP-mCherry* were genetically labeled by the addition of 5uM 4-OHT from 5-6dpf. Immediately following conversion, almost all mCherry+ cells express the radial glial cell marker GS. Yellow boxes indicate regions shown in lower panels. (B) Five weeks after 4-OHT addition, mCherry+ cells comprise radially oriented clones emanating from the posterior recess of the third ventricle (dashed line). (C-F) mCherry+ progeny 5 weeks after recombination include GS+ radial glia (C), HuC/D+ neurons (D), 5HT+ neurons (E), and *th2:gfp*+ dopaminergic neurons (F). Yellow boxes indicates region shown in lower panels, and double-labeled cells are indicated by red circles. Green signal in (C,D) is GFP from unconverted cells. Images are maximum intensity confocal Z-projections from ventral views of whole-mount brains. Scale bars = 10uM.

3.4.2. Wnt/ß-catenin signaling is dispensable for radial glial proliferation and formation of intermediate progenitors

We next tested whether Wnt/β-catenin signaling is necessary for the clonal expansion or neuronal differentiation of radial glia. Using heat shock-mediated expression of the secreted Wnt signaling inhibitor, Dkk1, after conversion of the Cre-labeled population with 4-OHT*, we examined* the effects on the lineage size and composition. After conversion from 5-6 dpf, *Tg(hsp701:dkk1-GFP)*^{w32} embryos were heat shocked once daily and fixed at 9 dpf. While Dkk1 expression effectively inhibited Wnt signaling by in-situ hybridization of the Wnt/ß-catenin target *sp5l* (Fig. 3.3), it did not result in a significant difference in overall clone size (Fig. 3.4), or in the percent of GS+ radial glia (Fig. 3.4B) or HuC/D+ neurons (Fig. 3.4C) in the lineage. These results suggest that radial glia can divide and undergo neuronal differentiation normally in the absence of Wnt pathway activity.

As an alternative method to inhibit Wnt signaling we examined zebrafish carrying a null mutation in *lef1,* a transcriptional mediator of the pathway (Wang et al., 2012). After conversion from 5-6 dpf and lineage analysis at 9 dpf , we found that *lef1* mutants also had no significant change in the percentage of GS+ radial glia (Fig. 3.4D) within labeled clones. As we reported previously, *lef1* is critically required to generate a specific subset of ventricular HuC/D+ neurons in the posterior recess (Wang et al., 2012). Our analysis confirmed that these *lef1*-dependent neurons arise from the radial glial lineage (Fig. 3.4E,F). However, they comprise only a small portion of radial glial progeny, and the number of nonventricular neurons was not decreased (Fig. 3.4E). Combined with the results of Dkk1 overexpression, these data lead us to conclude that Wnt/β-catenin

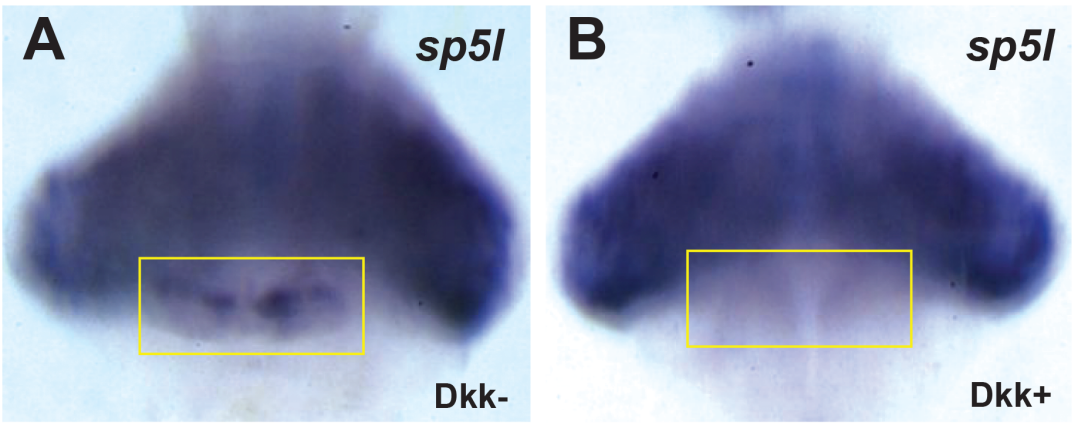
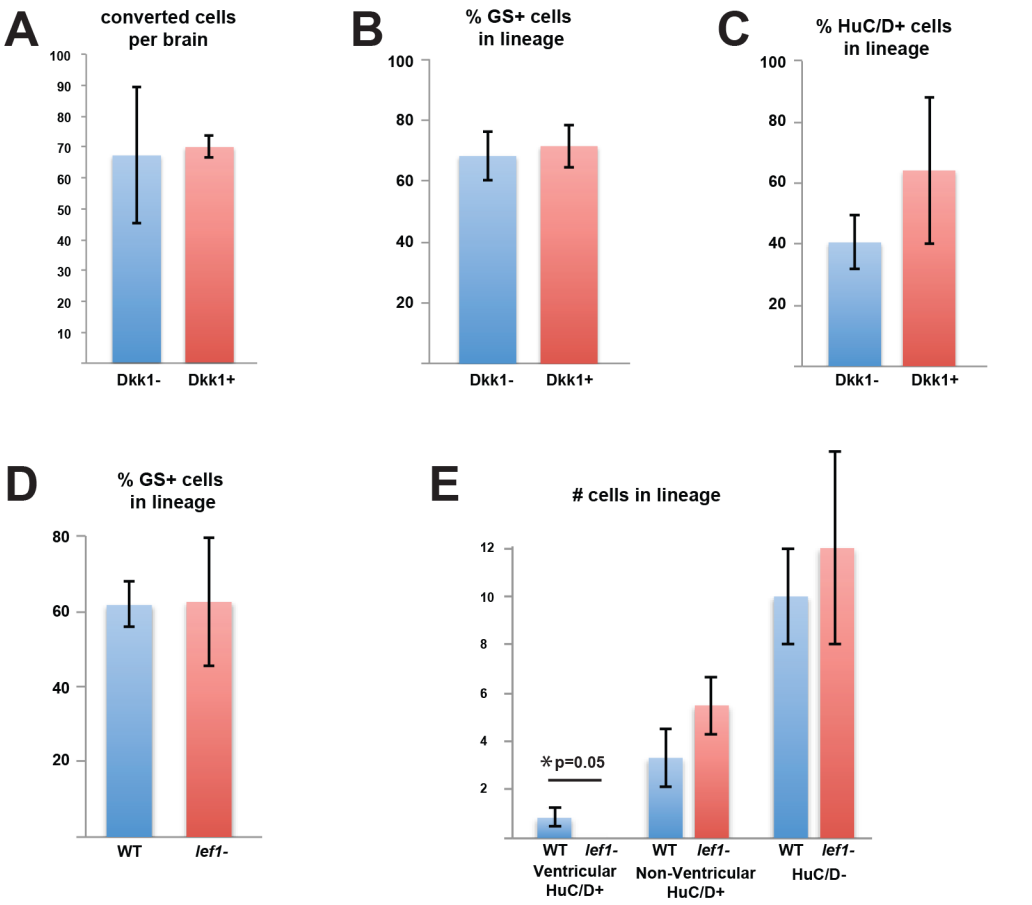
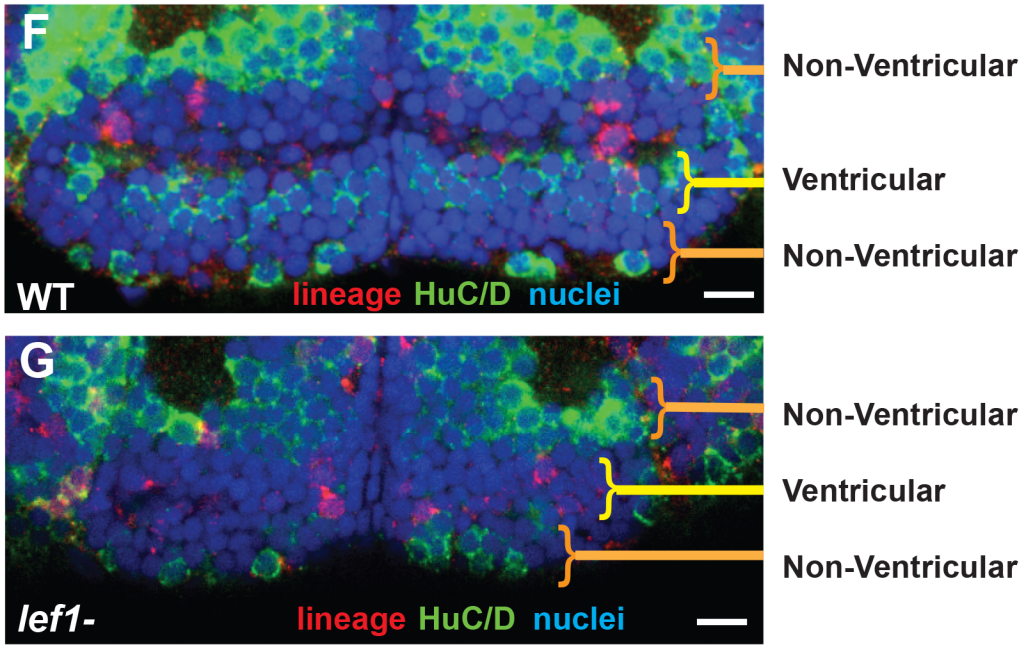


Figure 3.3. Hypothalamic expression of the Wnt target gene *sp5l* is eliminated by heat shock induction of Dkk1. (A) 5 dpf wild-type brain 3 hours after heat shock shows expression of *sp5l* mRNA in the posterior recess (yellow rectangle). (B) *hs:dkk1-GFP* brain 3 hours after heat shock shows loss of *sp5l* mRNA expression in the posterior recess (yellow rectangle). Images are ventral whole-mount views of dissected brains after in situ hybridization.

Figure 3.4. Wnt/ß-catenin signaling is not necessary for radial glial maintenance, proliferation, or general neuronal differentiation. (A-C) Following recombination of -*3her4.1:ERT2-Cre-ERT*+ progeny from 5-6 dpf, induction of Dkk1-GFP from 6-9 dpf does not affect the average number of labeled cells, N= 3 Dkk+, 5 wt brains (A), the percentage of GS+ cells, N= 6 Dkk+, 2 wt brains (B), or the percentage of HuC/D neurons, N= 3 Dkk+, 5 wt brains (C), in the lineage. (D) In *lef1* mutants there is no change in the percentage of GS+ cells after conversion from 5-6 dpf and analysis at 9 dpf, N= 4 *lef1-/-* 5 wt brains, (E-G) *lef1*-dependent ventricular neurons fail to arise from the radial glial lineage, while other progeny are unaffected, N= 3 *lef1-/-* 7 wt brains. Images are maximum intensity confocal Z-projections from ventral views of whole-mount brains.


A
converted cells per brain
100
90
80
70
60
50
40
30
20
10
Dkk1-
Dkk1+
B
% GS+ cells in lineage
100
80
60
40
20
Dkk1-
Dkk1+
C
% HuC/D+ cells in lineage
100
80
60
40
20
Dkk1-
Dkk1+
D
% GS+ cells in lineage
80
60
40
20
WT
lef1-
E
cells in lineage
12
10
8
6
4
2
*p=0.05
WT
lef1-
Ventricular HuC/D+
WT
lef1-
Non-Ventricular HuC/D+
WT
lef1-
HuC/D-


F
WT
lineage HuC/D nuclei
Non-Ventricular
Ventricular
Non-Ventricular
G
lef1-
lineage HuC/D nuclei
Non-Ventricular
Ventricular
Non-Ventricular

signaling is not necessary for radial glial proliferation self-renewal, or general neuronal differentiation in the postembryonic hypothalamus.

3.4.3. Partial genetic ablation of radial glia leads to increased proliferation of GS+ cells

To test whether hypothalamic radial glia show a regenerative response similar to other neural stem cell populations, we used the *Et(Gal4-VP16,myl7:gfp)*^{zc1066a} enhancer trap line in combination with the *Tg(UAS-E1b:NTR-mCherry)*^{jh17} effector line to express Nitroreductase (NTR) in radial glia, and thus ablate cells using metronidazole (MTZ)(Davison et al., 2007; Pisharath et al., 2007). After incubation in 1mM MTZ from 5-6 dpf, we observed partial ablation of radial glia (Fig. 3.5A,B), and the remaining cells, labeled either by GS or *her4.3:EGFP*, showed increased BrdU labeling from 7-8 dpf (Fig. 3.5C-F). This result suggested that radial glia can react to a decrease in their own population with a corresponding increase in self-renewal.

To determine if the proliferative response of radial glia to ablation requires Wnt/β-catenin signaling, we repeated our experiments in the presence of Dkk1 overexpression and in *lef1* mutants. In both cases, we observed the same level of BrdU incorporation in GS+ radial glia as in control animals. (Fig. 3.5G,H). These data indicate that just as in normal clonal expansion, the regeneration of hypothalamic radial glia is also Wnt/β-catenin independent.

3.4.4. Radial glia proliferate in response to genetic ablation of progeny

Because hypothalamic radial glia exhibit a proliferative response to partial ablation of their own population, we wanted to determine whether they respond similarly

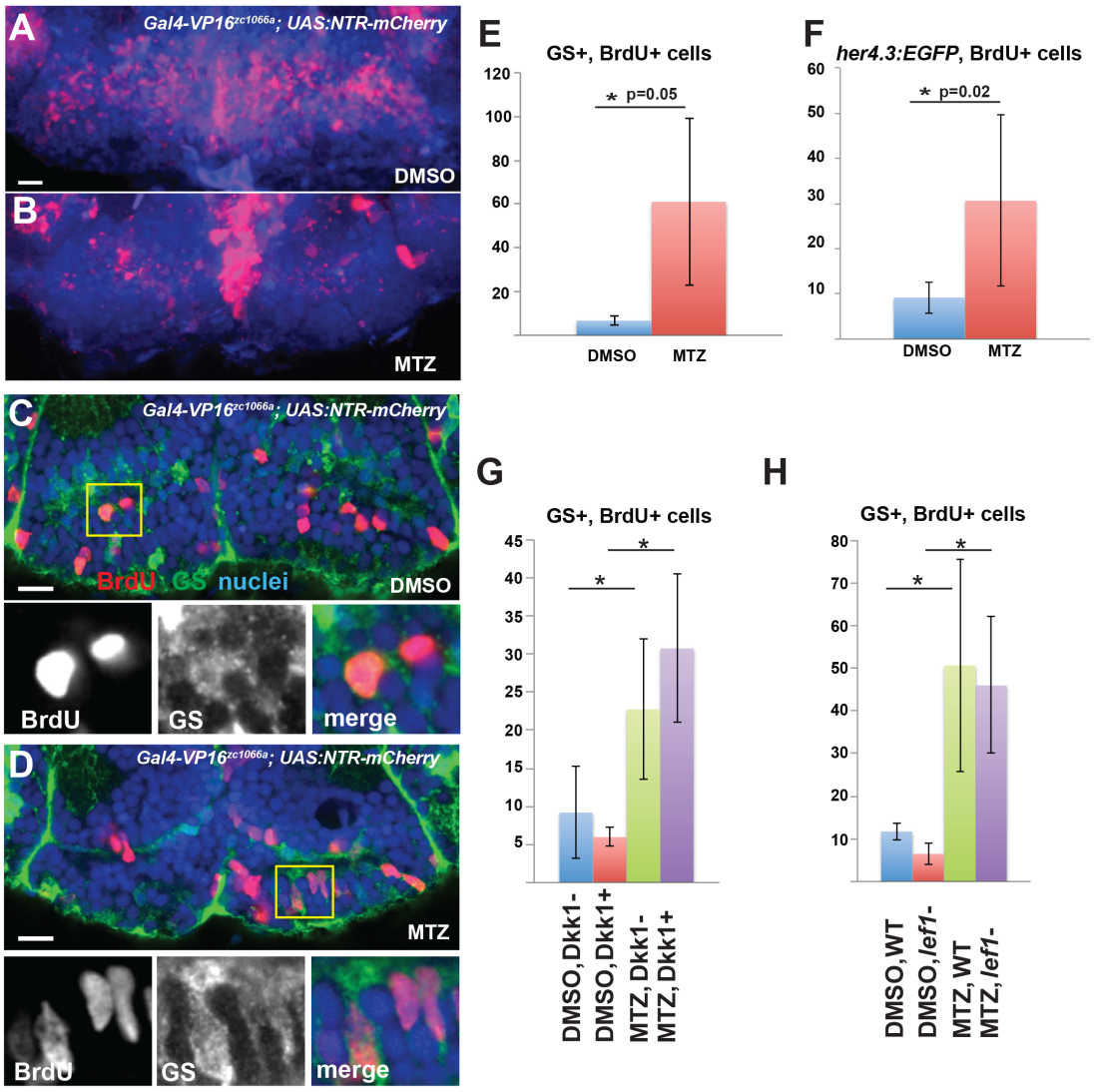


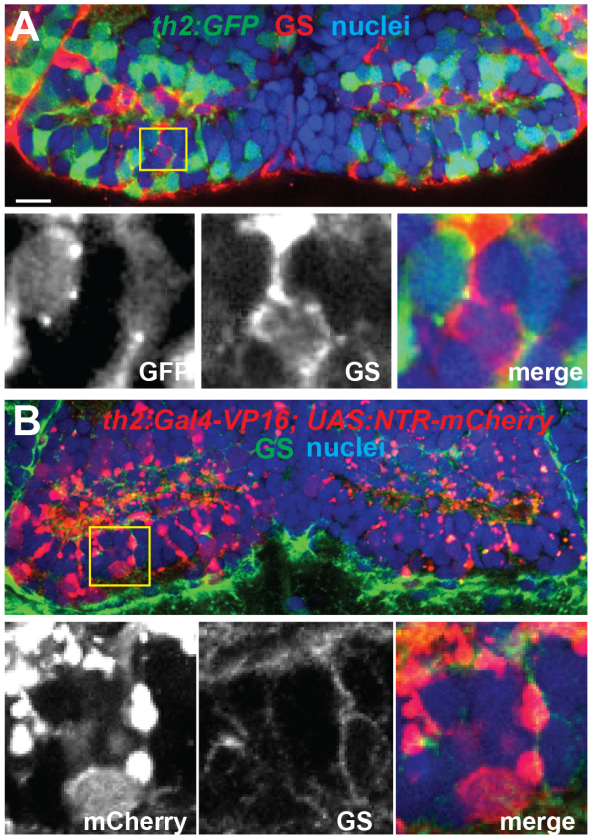
Figure 3.5. Hypothalamic radial glia respond to partial ablation by increasing proliferative activity. (A-B) Partial ablation of NTR-expressing radial glia (red) by incubation in 1mM MTZ from 5-6 dpf (C-F) After partial ablation from 5-6 dpf and BrdU labeling from 7-8 dpf (C-D, yellow box indicates region shown in lower panels), there is a significant increase in the number of BrdU+ radial glia marked by GS, N= 4 brains each DMSO and MTZ treated (E), and *her4.3:EGFP,* N= 5 DMSO treated, 3 MTZ treated brains, (F) expression. (G-H) Inhibition of Wnt signaling by Dkk1 expression, N= 5 DMSO treated Dkk+/- 4 MTZ treated Dkk+/- (G) or *lef1* mutation, N= 4 all conditions (H) does not affect the increase in BrdU labeling following partial ablation. Error bars = S.D., images are maximum intensity confocal Z-projections from ventral views of whole-mount brains. Scale bars 10uM.

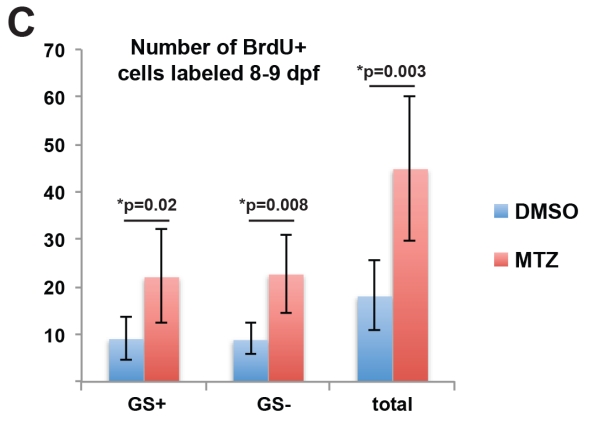
to the loss of a progeny cell type. Since we had observed that the lineage included cells labeled by the *th2* enhancer/promoter (Fig. 3.2F), and we found that the enhancer was not expressed in GS+ cells (Fig. 3.6 A), we used a transgenic line expressing *th2:Gal4-VP16* to drive *UAS:NTR-mCherry* expression (Fig. 3.6B). After incubation in 2.5mM MTZ from 5-6 dpf, we observed a significant increase in BrdU labeling of both GS+ and GS- cells at 8-9 dpf, 1 day later than with ablation of radial glia. (Fig. 3.6C). Despite an increase in the relative percentage of cells labeled by BrdU (Fig. 3.6D), the overall number of radial glia was decreased in MTZ-treated animals at 9 dpf (Fig. 3.6E), consistent with a transient increase in their differentiation.

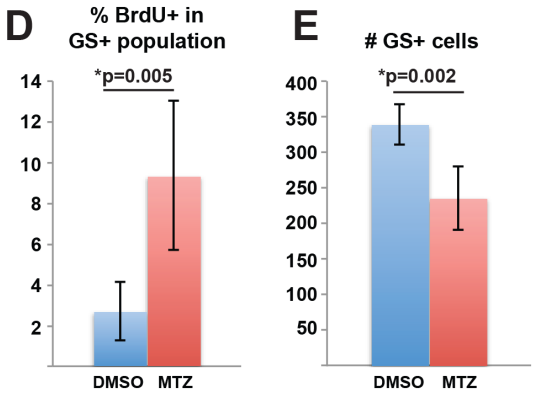
3.4.5. Wnt activation is sufficient to reduce the number of radial glia

Based on evidence that ectopic Wnt/ß-catenin signaling leads to the loss of radial glia (Wang et al., 2011a; Wang et al., 2012), we wanted to determine whether increased pathway activity promotes their differentiation. Following induction of *wnt8a* at 5 dpf using the heat shock-inducible transgenic line *Tg(hsp70l:wnt8a-GFP)*^{w34}, we observed a significant decrease in the number of GS+ cells at 6 dpf (Fig. 3.7A), confirming that this population of radial glia is lost after Wnt activation. To test the longer-term consequences of pathway activation while avoiding the lethality caused by continuous *wnt8a* expression, we incubated animals in 4uM 6BIO, a pharmacological inhibitor of GSK3β (Sato et al., 2004) from 6-9 dpf. This experiment also produced a significant decrease in GS+ radial glia (Fig. 3.7B), indicating a requirement for the absence of Wnt signaling in either self-renewal or maintenance.

Figure 3.6. Hypothalamic radial glia proliferate in response to ablation of dopaminergic progeny. (A-B) The *th2:GFP* (5 dpf, A) and *th2:Gal4* (9 dpf, B) transgenes do not label GS+ radial glia. Yellow boxes indicate regions shown in lower panels. (C) Ablation of *th2:Gal4*+ cells with MTZ at 5-6 dpf leads to an increase in BrdU+ labeling of GS+ radial glia and other cells from 8-9 dpf. (D) Ablation of *th2:Gal4*+ cells increases the percentage of GS+ radial glia labeled with BrdU from 8-9 dpf. (E) The overall number of GS+ cells is decreased at 9 dpf following ablation of *th2:Gal4*+ cells. Error bars = S.D., N=5 brains both DMSO and MTZ treated. Images are maximum intensity confocal Z-projections from ventral views of whole-mount brains. Scale bars 10uM.


A
th2:GFP GS nuclei
GFP
GS
merge
B
th2:Gal4-VP16; UAS:NTR-mCherry
GS nuclei
mCherry
GS
merge


C
Number of BrdU+ cells labeled 8-9 dpf
*p=0.02
*p=0.008
*p=0.003
70
60
50
40
30
20
10
GS+
GS-
total
DMSO
MTZ


D
% BrdU+ in GS+ population
*p=0.005
14
12
10
8
6
4
2
DMSO
MTZ
E
GS+ cells
*p=0.002
400
350
300
250
200
150
100
50
DMSO
MTZ

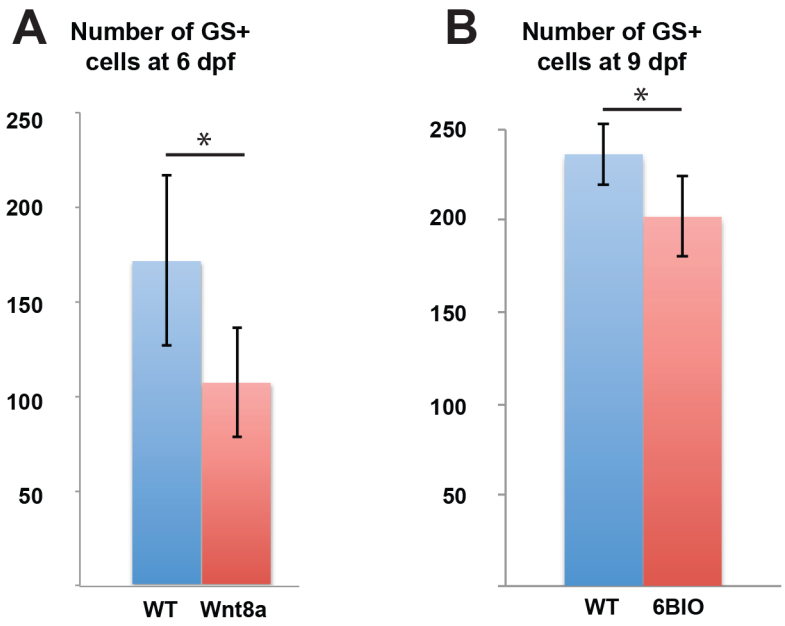


Figure 3.7. Wnt/β-Catenin signaling activity leads to a decrease in radial glia. (A) Induction of Wnt8a at 5 dpf leads leads to a decrease in GS+ radial glia at 6 dpf. (B) Addition of 4uM 6BIO, a GSK3ß inhibitor, daily from 6-9 dpf leads to a decrease in GS+ radial glia. N=4 WT brains and 5 experimental brains for each condition.

3.5. Discussion

3.5.1. Hypothalamic radial glia exhibit multiple features of neural stem cells

Our results demonstrate that hypothalamic radial glia in zebrafish are neural progenitors that can undergo a regenerative response, qualities that are hallmarks of a stem cell population. Because we were not able to follow the lineage of single cells, we cannot determine whether individual radial glia are multipotent with respect to neuronal progeny. However, the expansion that we observe in the lineage over a 5-week labeling period indicates that *her4.3*+ radial glia contribute significantly to the growth in size of

the posterior recess, and our marker analysis shows that the population generates several neuronal subtypes.

3.5.2. Wnt/β-catenin signaling is not necessary for hypothalamic radial glial self-renewal or expansion

It is well established that Wnt signaling regulates the rate of radial glial neurogenesis in the mammalian dentate gyrus (Jang et al., 2013; Lie et al., 2005; Seib et al., 2013; Zhang et al., 2008). These findings, as well as data from stem cell populations in other tissues (Holland et al., 2013; Nusse, 2008), have resulted in the hypothesis that Wnt/β-catenin signaling acts generally as a mitogen. However, our data indicate that pathway activity is not necessary for the proliferation of hypothalamic radial glia. In order to achieve the increase in population size we observe in our lineage analysis, these cells must undergo symmetric self-renewing divisions. In addition, regeneration requires symmetric self-renewing divisions at a higher frequency than in homeostatic neurogenesis. In contrast to evidence that Wnt/β-catenin signaling is necessary specifically for the symmetric amplification of telencephalic neural stem cells (Piccin and Morshead, 2011), our data show that pathway activity is not required for self-renewal or expansion of hypothalamic radial glia. It is likely that other signaling pathways drive radial glial proliferation in the zebrafish hypothalamus, including FGF (Kaslin et al., 2009; Robins et al., 2013), and Sonic Hedgehog (Dave et al., 2011; Komada, 2012; Shikata et al., 2011). We also find that Wnt/β-catenin signaling is not generally required for the neuronal differentiation of these cells, as opposed to its role in the mammalian dentate gyrus (Varela-Nallar and Inestrosa, 2013).

Our data also show that *lef1*-dependent neurons located near the hypothalamic ventricle (Wang et al., 2012) arise from radial glia, and further support a role for Wnt signaling in the differentiation of committed neural progenitors. Combined with other studies in the retina (Agathocleous et al., 2009), cerebral cortex (Munji et al., 2011; Zhang et al., 2014), hippocampus (Seib et al., 2013), and midbrain (Castelo-Branco et al., 2003), the majority of evidence suggests that in fact this may be the most widely conserved role for Wnt/β-catenin signaling in the central nervous system.

3.5.3. The absence of Wnt signaling is required for radial glial maintenance

We have found that Wnt/β-catenin signaling activation leads to depletion of GS+ hypothalamic radial glia. While Wnt ligands and reporters are expressed at high levels in the hypothalamic ventricular zone (Wang et al., 2012), the molecular mechanism by which pathway activity is inhibited in radial glia is unclear. Secreted antagonists such as Dkk or sFRPs could act extrinsically, or radial glia may fail to express the appropriate receptors to transduce Wnt activity. In the zebrafish telencephalon, Notch signaling maintains radial glial quiescence (Dong et al., 2012).

Further demonstrating the heterogeneity of radial glial populations throughout the central nervous system, a recent study driving activated β-catenin in radial glial progenitor cells with the *hGFAP* promoter has found that different progenitor populations vary dramatically in their interpretation of high Wnt/β-catenin signaling (Poschl et al., 2013). Ectopic activation of the Wnt pathway converts radial glia in the mammalian hypothalamus and cerebellum into alternative non-neural fates (Lee et al., 2012; Wang et al., 2012). Since it is possible that the lack of adult neurogenesis in mammals is partially

due to the loss of radial glia after embryogenesis, it is interesting to speculate that inhibition of Wnt/β-catenin signaling in radial glia prior to this timepoint might extend the capacity for regeneration.

3.6. Acknowledgements

R.N.D., A.D.M., and A.V.T. were supported by NIH T32 NS07067. R.I.D. was supported by NIH R01 NS082645. We thank the University of Utah Centralized Zebrafish Animal Resource (CZAR) for assistance with animal care, and Wenbiao Chen (Vanderbilt U.), Christian Mosimann (U. Zürich), and Ajay Chitnis (NICHD) for sharing zebrafish lines.

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CHAPTER 4

DISCUSSION

4.1. Overview

This dissertation has investigated the role of Wnt/β-Catenin signaling in the regulation of a potential stem cell population, radial glial cells of the posterior recess of the hypothalamus. I have performed a comprehensive expression analysis of *wnt* ligand expression during CNS development in the zebrafish (Chapter 2). This is the first description of this large gene family during vertebrate CNS development.

In addition, I have characterized a neural progenitor population in the zebrafish posterior recess of the hypothalamus, radial glial cells (Chapter 3). I have determined this population is able to generate different types of neurons during normal homeostatic neurogenesis and can support regeneration of itself as well as dopaminergic neurons. Multipotency and regenerative capacity are two key features of a stem cell population. I then went on to investigate the necessity of Wnt/β-Catenin signaling for the generation of neurons during normal homeostatic development and during regeneration of RGs. I found that Wnt/β-Catenin signaling is not necessary for these two RG cellular behaviors, indicating that RG populations are heterogeneous with respect to their dependence on Wnt/β-Catenin signaling to activate them or act as a mitogen. Finally, we have demonstrated that Wnt/β-Catenin signaling in fact needs to be attenuated for RGs to be maintained.

In this chapter, I will discuss the significance of our characterization of *wnt* gene expression patterns during CNS development. Radial glial potential and division properties during homeostasis and during a regenerative response will be then considered. I will go on to discuss the lack of necessity of Wnt/β-Catenin signaling on these RG 'activation' and will then speculate about potential regulators of RG 'activation' in the

hypothalamus. Wnt/β-Catenin signaling's general role in the promotion of differentiation generally will be presented as the most conserved and fundamental role this pathway plays postembryonically. Finally, the relevance of the role that we have discovered for Wnt/β-Catenin signaling, that it must be attenuated for RGs to be maintained, will be discussed.

4.2. *wnt* genes are expressed in overlapping domains in the CNS of the zebrafish

We have concluded the first comprehensive analysis of the expression of *wnt* genes in the vertebrate CNS. The analysis of multiple stages of CNS developmental will allow future investigators to determine which individual or combinations of *wnt* genes are potential drivers of proliferation, differentiation, cell migrations, and potentially innervation patterns (Bielen and Houart, 2014; Gordon et al., 2012; Holland et al., 2013; Veeman et al., 2003).

We have found that *wnt* genes are expressed in overlapping domains in the CNS, and importantly, *wnt11r*, *wnt16*, and *wnt8b* are all expressed in the PR of the hypothalamus. These genes could activate, or in the case of *wnt11r*, inhibit Wnt/β-Catenin signaling in the PR. To understand the consequence of loss of all Wnt ligands on the development and function of the PR, it would be necessary to generate triple mutants; however, single gene loss could generate a phenotype if there is a single Wnt signaling pathway being activated by a particular ligand. We have not investigated these mutants alone or in combination with regard to RG neurogenic potential or regenerative responses. In this particular case, there exist mutant alleles for both *wnt11r* and *wnt16*. The creation of a *wnt8b* mutant allele using TALEN or CRISPR/Cas9 technology

(Dahlem et al., 2012; Hruscha and Schmid, 2015) would allow the combination of all three alleles in a single animal. One can already appreciate the complexity of this simple loss of function strategy. Alternative methods for studying Wnt/β-Catenin signaling loss of function are recommended as a result. These include mutating receptors that transduce the pathway, such as *frizzled* or *low density lipoprotein receptor-related protein* 5 or *6* genes, or other critical components of Wnt/β-Catenin signaling. Such targets will likely present different problems for loss of function analysis of Wnt/β-Catenin signaling, including early lethality and affects to other pathways besides Wnt/β-Catenin signaling. Finally, inducible expression of Wnt/β-Catenin inhibitors, for example from the *Tg(hsp70:dkk1-GFP)* transgenic line or pharmacological manipulation, will likely therefore offer the best methods for Wnt/β-Catenin signaling manipulations, as these methods can be utilized subsequent to early development (Zimmerman et al., 2012).

4.3. Radial glial cells are multipotent neural progenitors and support regeneration

4.3.1. Hypothalamic RG are self-renewing multipotent neural progenitors

I was interested in determining the potential of RGs in the hypothalamus. I used the *her4* promoter to genetically label RGs after embryogenesis was complete. The hypothalamic Her4+ lineage expands from a small number of converted RGs per animal into a large number of RGs and neurons. This population generates serotonergic, dopaminergic, and hucd+ neurons while expanding the number of RGs. The expansion of the RG population from a small number of RGs indicates that at some frequency these RGs must divide symmetrically to expand their population. As this tissue is continually growing, RGs need to expand their population to maintain homeostatic tissue

composition. It does appear that individual RGs are in fact multipotent, generating multiple neural subtypes, as we can see that the composition of the Her4+ lineage contains serotonergic, dopaminergic, and HuC/D+ neurons in the same proportion as the tissue as a whole. We could be observing the presence of two differently lineage restricted, but adjacent, RGs that were converted to express mCherry. Only in such a case would we observe intermingling of neural subtypes within a contiguous Her4 lineage population if RGs were not individually multipotent. This is unlikely given that we have observed multiple brains with large Her4+ lineages that are composed of HuCD+ neurons, 5HT neurons, and dopaminergic neurons, all qualitatively present in the Her4+ lineage at the normal proportion these cells are present in the hypothalamus. Only after labeling individual RGs will we be able to determine the true nature of individual RG potential. This could be performed using a lower dose of 4-OHT than used in this work or by using the *her4.1* promoter to drive expression of an inducible Cre-recombinase in combinase with a zebrabow construct to allow single RGs to be labeled by a spectrally unique combination of fluorophores.

4.3.2. RG cells respond to ablation of RG cells by entering the cell cycle

To test the regenerative potential of RGs we used a low dose of MTZ in *Tg(Et(Gal4-VP16,myl7:gfp)*^{zc1066a} *;UAS-E1b:NTR-mCherry)*^{jh17} animals to partially ablate RGs. We then use BrdU to measure the proliferative response of RGs. We show that there's an increase in BrdU incorporation specifically in RGs 1-2dpf after MTZ mediated ablations, which is an indirect measure of proliferation. BrdU is incorporated into the DNA of cells in the synthesis (S) phase of the cell cycle. Therefore, BrdU+ cells

are either cells that are currently in S-phase of the cell cycle or were in S-phase during the BrdU pulse and subsequently underwent mitosis yielding a BrdU+ doublet. In addition, BrdU is incorporated into DNA of cells undergoing DNA damage repair. The increased BrdU+ cell number after ablations likely reflects the tissue integrating the altered composition of the PR, after dead RG cells have been cleared, and signaling to RGs to divide to replenish the tissue.

We have not absolutely demonstrated that RGs are dividing symmetrically. Future investigations into the nature of RG's proliferative response after partial ablation should include the analysis of PAR protein distribution in dividing RGs. Par3, which is assymetrically localized in telencephalic RGs during early development and specifies self-renewal (Dong et al., 2012), might be distributed evenly RGs that have divided in response to RG partial ablation. This would be strong evidence that RGs are actually being expanded through symmetric divisions after partial ablation of this population. In addition, we have not shown that the full cadre of RGs is replenished subsequent to RG ablations. This could be easily investigated by counting the size of the RG population before and at different times RG ablations.

4.3.3. RG cells respond to dopaminergic ablations by entering the cell cycle

We next were interested to determine if RGs would support a regenerative response after dopaminergic neurons were ablated using the *th2* promoter. After *th2* ablations, there is an increase in BrdU+ RGs 2-3 days after ablation. This response is 1 day after that seen during RG ablations. It is likely that the delay in proliferation of RGs is due to the presence of an intermediate progenitor population responding to generate

dopaminergic neurons. After these intermediate progenitors are lost by dividing or differentiating to generate dopaminergic neurons, RGs divide to restore these intermediate progenitors. Support for this model comes from the fact that there is a significant increase in the BrdU+, GS-negative cells 2-3 days after ablation as well, which could represent this putative intermediate progenitor population.

We have not specifically demonstrated that individual RGs are truly asymmetrically dividing cells. We believe this is the case due to the presence of radial clones emanating from the ventricle- likely derived from a single RG cell. However, due to the expansion of the RG population itself through time and due to RGs regenerative response, we know that RGs must divide symmetrically as well. The data demonstrate that the population, however, is a self-renewing, multipotent population. Utilizing brainbow technology (Pan et al., 2013) would allow us to label individual RG cells. If individual clones are expanded while others are lost, it would support the model that RGs divide symmetrically and are stochastically renewed or differentiation. However, if individual clone size remained constant, it would support the model that RGs divide asymmetrically to generate differentiating progeny while self-renewing.

It is interesting to speculate that only after tissue clearance does the PR somehow 'sense' the lack of a particular cell type and signal RGs to proliferate appropriately to regain homeostasis. This could be tested by combining our RG specific gal4 line or *th2* gal4 line and our *her4.1* lineage transgenes to create Tg*(ubi:loxP-eGFP-loxP-mCherry ; -3her4.1:ERT2-Cre-ERT2 ; Gal4-VP16,myl7:gfp*^{zc1066a}*; UAS-E1b:NTR-mCherry)* or *Tg(-3her4.1:ERT2-Cre-ERT2;ubi:loxP-eGFP-loxP-mCherry; th2:Gal4-VP16 ; UAS-E1b:NTR-mCherry)*. After postembryonic conversion of the *her4.1* lineage to express

mCherry, RGs or TH2 neurons could be partially ablated or ablated, respectively. After these ablations the nature of the RG progeny generated during the proliferative response could be analyzed using cell type specific markers. After ablation of amacrine and ganglion cells in the mammalian retina Mueller glia dedifferentiate and generate amacrine cells, indicating retinal RG cells can sense the presence or absence of progeny cells and respond accordingly (Karl et al., 2008). It is unclear why ganglion cells are not replaced in this paradigm.

4.4. Wnt/β-catenin signaling is not necessary for RG proliferation or maintenance

4.4.1 Wnt/β-catenin signaling is not necessary for RG activation, homeostatic neurogenesis or maintenance

A cell in quiescence is not currently in the cell cycle, although is also not post-mitotic. The signaling that stimulates the transition from this cell cycle arrested state to actively cycling RG in the hypothalamus is unknown. The well-characterized radial glial stem cell population in the dentate gyrus of the hippocampus utilizes Wnt/β-catenin signaling as a mitogen (Jang et al., 2013; Lie et al., 2005; Seib et al., 2013; Zhang et al., 2008). In addition, Wnt/β-catenin signaling is necessary specifically for the symmetric amplification of stem cells in the SZV and in-vitro (Piccin and Morshead, 2011). As a result, we hypothesized that Wnt signaling functions to stimulate RG cells in the hypothalamus to enter the cell cycle to either expand their population through symmetric divisions or to generate neurons. To determine the role Wnt/β-catenin signaling plays in RG maintenance, homeostatic neurogenesis, and self-renewal we combined genetic labeling of RGs with Wnt/β-catenin signaling knockdown via induction of *dkk1* and

mutation of *lef1*. We have determined that hypothalamic radial glial cells do not require Wnt/β-catenin signaling for self-renewal, maintenance, or expansion during normal growth. Because *lef1* mutants do not generate ventricular HuC/D neurons but do possess HuC/D neurons in other nonventricular zones of the PR, it appears that similar to the hair follicle niche, Wnt signaling is completely dispensible for RG proliferation but is required for downstream differentiation (Blanpain and Fuchs, 2009; Lien et al., 2014).

4.4.2. Wnt/β-catenin signaling is not necessary for regeneration of RGs

We next investigated if Wnt/β-catenin signaling is necessary for regenerative proliferation of RGs after partial ablation of RGs using Tg(*(UAS-E1b:NTR-mCherry)*^{jh17} *Et(Gal4-VP16,myl7:gfp)*$^{zc1066a)}$ and a low dose of MTZ. In spite of reports that Wnt/β-catenin signaling is necessary specifically for the symmetric amplification of stem cells in the SZV and in-vitro (Piccin and Morshead, 2011), including during a regenerative response in-vivo, we have demonstrated that Wnt/β-catenin signaling is not required for RG regeneration. During telencephalic neural regeneration in the zebrafish, *her4.1*+ RGs are stimulated to increase proliferation and generate neurons in response to injury-specific-induction of *gata3* (Kizil et al., 2012b). Interestingly, FGF signaling is necessary for the induction of *gata3* after injury. FGF signaling drives RG proliferation in telencephalic development (Ganz et al., 2010; Kaslin et al., 2009); therefore, this aspect of development is recapitulated during regeneration yet downstream of FGF signaling *gata3* expression is a unique and critical regulator of regeneration. It is likely that similar to that observed in the telencephalon, some aspects of the homeostatic neurogenesis mechanisms are conserved in the regeneration of RGs in the posterior recess, yet others

are likely specific to the regeneration process. FGF signaling and *gata3* would be good candidate regulators of RG regeneration in the hypothalamus.

4.5. Potential regulators of RG cell activation in the posterior recess

Candidate regulators of RG activation and proliferation include those used by RGs and CNS stem cells. Fibroblast Growth Factor (FGF) signaling functions as a mitogen in cerebellar Bergmann glia (Kaslin et al., 2009), Sonic Hedgehog (SHH) signaling fine-tunes the cell cycle of embryonic and adult neural progenitors (Komada, 2012), while in the retina, Ascl1 reprograms Mueller glia to re-enter the cell cycle and become neural progenitors (Pollak et al., 2013). Ascl1 is not required for hippocampal RG activation (Lugert et al., 2012), further demonstrating the heterogeneous nature of RG populations.

It has been observed that the cell cycle of stem cells is functionally different than the cell cycle of more differentiated cells, and this has been hypothesized to be a functional mode of regulating the potential of stem cells (Gotz and Huttner, 2005; Roccio et al., 2013). Shh signaling perturbations have shown that Shh activation leads to the transition of slow-cycling neural stem cells to fast-cycling neural progenitor cells (Komada et al., 2008) in the developing cortex. Indeed, Shh receptor expression is restricted to the stem cell population, astrocytic B-cells, in the SVZ, and *mGfap-Smo CKOs*, in which Smoothened is knocked out of B-cells, caused a progressive depletion of stem cells in the SVZ and a reduction in the number of transit amplifying cells and olfactory bulb neurons generated. This loss of stem cells was likely due to conversion of stem cells to fully differentiated astrocytes (Petrova et al., 2013), which could be the

result of dysregulation of the cell cycle by perturbed SHH signaling. Noteably, in *mGfap-Gli3;Smo CKOs*, in which the repressive Shh receptor *gli3* is also mutated, there is a partial rescue of the deficits seen in *mGfap-Smo CKOs*, indicating the levels of Shh signaling are critical for the correct regulation of proliferation of stem cells in the SVZ. The tightly controlled activation of Shh signaling in RGs could activate them to enter the cell cycle. SHH reporters are present in the hypothalamus (*Tg(ptch2:mcherry)* (data not shown). In the presence of pharmacological inhibitors of SHH signaling, it would be interesting to see if RGs in the hypothalamus have altered differentiation or proliferative potential during homeostatic neurogenesis and during regenerative responses.

There is evidence that Notch signaling functions in asymmetrically dividing RGs in the telencephalon to keep one progeny cell self-renewing as a quiescent RG and the other progeny cell differentiating through activation of neurogenic cascades including *ascl1* and *neurogenin* expression (Chapouton et al., 2010). In addition, it was shown that in telencephalic development RGs are signaled to have active Notch signaling through the expression of *delta* ligands by their progeny cells that remain adjacent to them (Dong et al., 2012). RG cells could simply be activated by the eventual loss of these Delta ligands presented to them by their progeny. Upon cell loss during ablation, or cell migration during differentiation, Notch signaling might be attenuated in the parental RG cell, which would then push the RG into a proliferative state. In this way, the activation of RGs would be indirectly regulated by the absence of progeny derived inhibitory signals (Delta ligands). We have used the *her4.1* promoter to drive expression of Cre recombinase, thus labeling RGs in the posterior recess that have active Notch signaling and are being held in a quiescent state. RGs labeled by expression of *her4.1,* however,

could be a diverse group. Interestingly, Hes1 (a *her4* family member) and Neurogenin2 were shown to induce two contrasting cell cycle arrest states in early and late G1, ultimately leading to reversible quiescence or irreversible differentiation, respectively. This model does a good job of explaining simple asymmetric self-renewing divisions of RG cells but cannot account for RG expansions. This could be possible if both daughter cells were induced to have a high level of Hes1 expression, either by exposure to high levels of Delta-ligands to activate high Notch levels in both progeny cells or by Ngn2 inhibition induced by other unidentified pathways. It is therefore necessary for the organism to utilize another way to regulate cellular division or a way to modify Notch signaling to generate two daughters with high Notch signaling, that will maintain expression of *her4/hes5* and presumably renew as RGs. It was recently shown that the transcription factor *fezf2* interprets notch activity, functionally affecting cell fate decisions in NSCs (Berberoglu et al., 2014); this transcription factor could be involved in RG maintenance in the PR.

We have demonstrated that Wnt/β-catenin signaling does not drive symmetric divisions, but we can hypothesize that potentially Wnt/PCP signaling might. Muscle stem cells, the satellite cells, are stimulated to symmetrically divide and self-renew through wnt7/Frizzled7 signaling during muscle regeneration (Le Grand et al., 2009). *wnt11r* is expressed in the posterior recess of the hypothalamus in zebrafish and involved in β-catenin-independent Wnt signaling pathways during neural crest cell migration and during neuromuscular junction innervation (Banerjee et al., 2011; Gordon et al., 2012). The presence of a zebrafish *wnt11r* mutant offers the ability to test the necessity of this ligand in RG symmetrical divisions- both by crossing this mutant into her4:ert2-cre-ert2;

ubi:switch and gal4:zc1066a; uas:ntr-mcherry to observe the expansion of the her4+ RGs in a radial clone during normal homeostatic neurogenesis and during regeneration of RGs after partial ablation.

4.6. Wnt/β-catenin signaling supports differentiation

Although it is now clear that there is little evidence to support a universal role of Wnt/β-catenin signaling in the regulation of neural stem/progenitor cells, there is compelling evidence to support a general role for Wnt/β-catenin signaling in the promotion of neural differentiation, either through the proliferation of transit amplifying cells, or through the activation of genes essential for neural function. We suggest that in fact, the most conserved role for Wnt/β-catenin signaling across nervous system proliferative zones is to support the differentiation of progeny neurons, and not to signal as a mitogen in the stem cell populations. Support for this hypothesis can be found by observing the many tissues with documented roles for Wnt/β-catenin in promotion of neural differentiation, such as the retina (Agathocleous et al., 2009), cerebral cortex (Munji et al., 2011; Zhang et al., 2014), hypothalamus (Lee et al., 2006), hippocampus (Seib et al., 2013), and midbrain (Castelo-Branco et al., 2003). Indeed direct targets of Wnt/β-catenin signaling in thalamic neurons, including gaba receptors, calretinin, and voltage-gated ion channels (Wisniewska et al., 2012). Finally, Wnt/β-catenin signaling is required for the transition from proliferating neural progenitor cells to postmitotic differentiating neurons in *platynereis dumerili* (Demilly et al., 2013), indicating this might be an ancient and conserved function.

In addition to driving the expression of neural genes in postmitotic neurons, a complex interplay between the cell cycle and Wnt/β-catenin signaling is likely involved in transit amplifying populations (Niehrs and Acebron, 2012). Wnt/β-catenin signaling is activated when stem cells are differentiating and their progeny are rapidly dividing (Lowry et al., 2005) in the hair follicle, but completely dispensable for the division of stem cells in this population.

4.7. Wnt signaling needs to be inhibited to maintain RGs

Although we have demonstrated conclusively that Wnt/β-catenin signaling is not necessary for RG maintenance or division, it is interesting to note that Wnt/β-catenin is sufficient to drive RGs to alternate fates. A recent study driving activated β-catenin in radial glial progenitor cells with the *hGFAP* promoter has found that different progenitor populations vary dramatically in their interpretation of high Wnt/β-catenin signaling (Poschl et al., 2013). While DG RGs proliferated at a higher rate than normal, Bergmann glia in the cerebellum were driven into alternate fates and not maintained as RGs, and finally, SVZ astrocytes where initially amplified before they were lost without differentiation. We have found, in addition, that Wnt/β-catenin signaling activation leads to depletion of RGs in the hypothalamus. As RGs in the mammalian hypothalamus turn into astrocytic cells (Wang et al., 2012) and Bergmann glia adopt astrocytic character (Wang et al., 2011a), we strongly suspect that RGs in the zebrafish hypothalamus will similarly adopt a non-RG fate; however, as zebrafish do not possess astrocytes, it will be interesting to see if these RGs maintain expression of RG markers and yet adopt a morphology reminiscent of mammalian astrocytes. It is possible that the widespread

dearth of adult neurogenesis in mammals is due to the widespread loss of RG cells as development ends. It would be interesting to see if RGs are maintained in mammalian nervous system tissues if Wnt/β-catenin signaling where attenuated in RGs specifically. One way to test this hypothesis would be to express a repressor TCF factor, for example *tcf3*, in RGs. This might allow the nervous system of mammals to repair injury.

4.8. Mechanisms of inhibition of Wnt/β-catenin signaling in radial glial cells

Wnt/β-catenin signaling ligands and reporters are expressed at high levels in the posterior recess. Radial glial cells, however, have low levels of Wnt/β-catenin signaling. The molecular mechanism by which Wnt/β-catenin signaling is inhibited in these cells is currently unknown. We know ligands are present in the posterior recess, and next to many RGs are cells with high levels of Wnt signaling reporters. Clearly, these RG cells must either not be competent to respond to this signal (not have appropriate receptors) or actively inhibited from transducing Wnt signaling. A number of possibilities exist that could inhibit Wnt/β-catenin signaling specifically in RG cells. Secreted antagonists, for example *dkk1* or *secreted frizzled related proteins*, could be acting on radial glial cells to inhibit Wnt/β-catenin signaling or RG cells might not express the right complement of *frizzled* or *lrp* receptors to transduce Wnt activity. In the telencephalon of the zebrafish, intralineage Notch signaling keeps RG cells quiescent (Dong et al., 2012). Progeny cells of dividing RGs express Delta ligands that act on their parent cell to activate Notch signaling which functions in RGs to keep them quiescent. In the same way, Wnt/β-catenin signaling could be inhibited in RGs by the expression of Wnt inhibitors, such as *sfrp* or *dkk1* genes, by neighboring cells.

4.9. Summary

Interestingly, but perhaps not surprisingly, we have uncovered novel ways in which an RG progenitor population interprets Wnt/β-catenin signaling during development and regeneration. In particular, we have found that Wnt/β-catenin signaling is not required for these processes but is sufficient to drive RGs in the posterior recess into other fates. The finding that Wnt/β-catenin signaling is not necessary for neural differentiation but is sufficient to drive RGs into alternative fates is a relatively rare relationship for a signaling pathway to have with a population. It could imply that other pathways might work in parallel with Wnt/β-catenin signaling to activate RG cell differentiation, but only if RGs are transformed into neurons during conditions of Wnt signaling activation in the zebrafish hypothalamus. Alternatively, this RG transformation could be the result of extra-physiological levels of Wnt signaling, with little biological relevance beyond understanding the potential of RG populations in pathophysiological conditions such as Wnt mutations potentially generating cancer. Redundant regulation of RGs differentiation by multiple pathways offers tight control of hypothalamic neurogenesis and ensures that the circuits that are impacted by the addition of new neurons are not easily perturbed. Due to the critical nature of the biological processes that the hypothalamus serves, such as sexual maturity, energy homeostasis, stress responses, and fear or aggression, this tight regulation might be necessary to ensure the animal's behavior is not negatively affected by misregulation of hypothalamic neurogenesis.

4.10. References

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